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14. ABSTRACT The overall goal of this proposal is to develop fully synthetic vaccines against respiratory infections using novel nanotechnology platforms based on safe and degradable adjuvant polymer systems that enhance antigen presentation and stimulate immunity. The proposal focuses on achieving the following specific technical objectives: 1) Develop molecular methods for intervention strategies employing novel synthetic nanovaccine platforms encapsulating DNA and protein/peptide antigens that elicit immune response against influenza H5N1; 2) Test the efficacy of nanovaccines-based intervention regimens against influenza H5N1 in animal models. 3) Adopt intervention strategies using most promising nanovaccine platforms to other respiratory infections, such as pulmonary F. tularemia, and test the nanovaccines efficacy in animal models (unfunded option). Focusing on respiratory infections, the leading cause of outpatient illness and a major cause of infectious disease hospitalization in U.S. military personnel, has the potential to develop effective, safe and affordable synthetic vaccines.					
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- 1. INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The overall goal of this proposal was to develop fully synthetic vaccines against respiratory infections using novel nanotechnology platforms based on safe and degradable adjuvant polymer systems that enhance antigen presentation and stimulate immunity. Three novel versatile technology platforms were evaluated: a) biodegradable polyanhydride nanospheres (BPN) carrying a polypeptide/protein antigen (Platform A); b) polymeric micelles of Pluronic block copolymer as DNA vaccine adjuvant (Platform B); and c) block ionomer complexes (BIC) for targeted delivery of DNA (or protein) antigen to the antigen presenting cells (APCs) (Platform C). The central hypothesis was that these polymeric nanoscale delivery systems could provide versatile platforms for development of effective, safe, and cost-efficient vaccines. The materials used in these platforms demonstrated immunomodulatory capabilities and can be targeted to specific populations of immune cells to elicit most efficient immune response. The proposal was focusing on achieving the following specific technical objectives: 1) Develop molecular methods for intervention strategies employing novel synthetic nanovaccine platforms encapsulating DNA and protein/peptide antigens that elicit immune response against influenza H5N1; 2) Test the efficacy of nanovaccines-based intervention regimens against influenza H5N1 in animal models. The best technology or their combination will be determined for future development.

- 2. KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Nanovaccine, polymers, influenza
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- 3. ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

**What were the major goals of the project?**

*List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.*

The following tasks and timeline were proposed.

**YEAR 1**

Task 1: Establish project: a) the project teams will be assembled at both sites of project performance; b) new essential equipment will be acquired; and c) IACUC and IBC protocols will be submitted and approved. ACCOMPLISHED

Task 2: Produce DNA plasmids expressing viral antigens (Platform A, B, & C). ACCOMPLISHED

Task 3: Produce recombinant influenza A virus H5N1 proteins (Platform A & C). ACCOMPLISHED

Task 4: Synthesize cationic copolymers for BIC/DNA compositions (Platform C). ACCOMPLISHED



## YEAR 2

Task 1: Synthesize a library of polyanhydride copolymers (Platform A). ACCOMPLISHED

Task 2: Prepare and characterize targeted BIC/DNA compositions (Platform C). ACCOMPLISHED

Task 3: Determine optimal Pluronic/DNA and BIC/DNA compositions to maximize levels and duration of transgene expression after administration in skeletal muscle in a mouse (Platform B & C). ACCOMPLISHED

## YEAR 3

Task 1: Determine optimal antigen-containing BPN that activate dendritic cells (DCs) (Platform A). ACCOMPLISHED

Task 2: Use optimal Pluronic/DNA and BIC/DNA compositions determined in Y02 to achieve maximal antigen expression (Platform B & C). ACCOMPLISHED

Task 3: Establish/adapt model for aerosol virus challenge; test viability/infectivity of virus preparations. ACCOMPLISHED

Task 4: Determine optimal antibody response generated by nanovaccine developed in each platform. ACCOMPLISHED

## YEAR 4

Task 1: Evaluate vaccines, assess immunogenicity in vivo, and determine the lead nanovaccine configurations for aerosol virus challenge studies. ACCOMPLISHED

Task 2: Determine vaccine that protect against H5N1 influenza virus in challenge studies. ACCOMPLISHED

### **What was accomplished under these goals?**

*For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.*

The tasks accomplished are described below:

### **Platform A**

#### **Year 1**

In respect of the design DNA plasmids encoding H5N1 HA and NA antigens:

1. pHW2000 A plasmid (from St. Jude's influenza seed virus 163243) containing the gene for H5 HA protein of influenza A/Whooper Swan/Mongolia/244/05 was used for PCR overlap extension reaction.

-Overlap extension allowed for reversion of the HA cleavage location allowing the protein to be more structurally similar to wild type.

2. H5 HA gene was cloned into a pMT/BiP/V5-His (Invitrogen) protein expression plasmid.

- The plasmid has been sequenced and shown 100% sequence homology of the H5 sequence with the field isolate virus.

-BiP is a secretion sequence allowing release of the protein when induced with CuSO<sub>4</sub>.

Because H5 would be glycosylated when produced by eukaryotic cells, we have transfected Drosophila S2 cells rather than to express the protein in bacteria.

3. H5 pMT/BiP/V5-His was co-transfected with pCoBlast plasmid using MaxFect transfection reagent into a S2 Drosophila cell line.

-pCoBlast is blasticidin resistance plasmid allowing for blasticidin selection to be performed post transfection.

4. Two week blasticidin selection of four cell “colonies” was performed.

5. Four cell colonies were induced for 3 days using 700  $\mu$ M of CuSO<sub>4</sub>.

6. Western blots of cell supernatants and cell lysate were performed using hyperimmunized pig sera.

-Pigs immunized with 1024 hemmagglutination units (HAU) of inactivated virus and given booster injections 2 and 4 wks post inoculation.

In support of milestone related to production and purification of recombinant H5N1 protein:

1. Scale up of transfected S2 cells has been accomplished.

2. Purification of recombinant H5 protein by utilizing the 6xHis tag on the C-terminus of the protein is being carried out using a nickel-chelating resin.

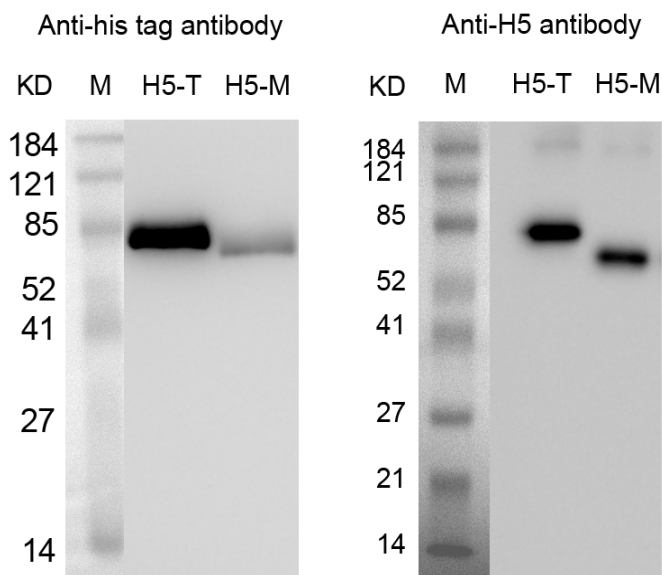
Polyanhydride copolymers based on sebacic acid (SA), 1,6-(*p*-carboxyphenoxy)hexane (CPH), and 1,8-(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) were synthesized by melt polycondensation at 180 C and 0.3 Torr vacuum for 90 minutes. Copolymers based on CPH:SA and CPTEG:CPH were synthesized at various copolymer compositions. The copolymers were characterized with <sup>1</sup>H nuclear magnetic resonance (NMR) spectroscopy, Fourier transform infrared (FTIR) spectroscopy, and differential scanning calorimetry (DSC). End group analysis of the NMR spectra was used to determine molecular weight, copolymer chemistry, and chemical structure with a Varian VXR 300 MHz spectrometer (Varian Inc., Palo Alto, CA). Deuterated chloroform was used to dissolve the samples and chemical shifts were calibrated with respect to the chloroform peak ( $\delta$ = 7.26 ppm). The surface chemistry of the polymer libraries was characterized using FTIR spectroscopy (Nicolet 6700 FTIR spectrometer, Thermo Fisher Scientific). The thermal properties were measured using DSC.

Library synthesis: A linearly varying library in molar composition of CPH:SA prepolymer was deposited using an automated robotic apparatus. The prepolymer library was polymerized at 0.3 torr vacuum and the desired temperature (180 °C for CPH:SA and 140 °C for CPTEG:CPH) for 1.5 h resulting in a discrete library of CPH:SA (poly (SA), 25:75 CPH:SA, 50:50 CPH:SA, 75:25 CPH:SA, and poly (CPH)) and CPTEG:CPH (60:40 CPTEG:CPH, 50:50 CPTEG:CPH, 40:60 CPTEG:CPH, 30:70 CPTEG:CPH, 20:80 CPTEG:CPH and 10:90 CPTEG:CPH) copolymers in replicates of four.

**Nanoparticles:** The discrete polymer libraries were dissolved in methylene chloride. The polymer solution was sonicated for 30 s at 40 Hz and the resulting solution was precipitated into a non-solvent (pentane) to create nanoparticles and dried in a vacuum chamber (CPH:SA) or undergo rapid filtration (CPTEG:CPH).

**Polymer and nanoparticle characterization:** The discrete polymer libraries were characterized with  $^1\text{H}$  nuclear magnetic resonance (NMR) spectroscopy and high throughput Fourier transform infrared (FTIR) spectroscopy. End group analysis of the NMR spectra was used to determine molecular weight, copolymer chemistry, and chemical structure with a Varian VXR 300 MHz spectrometer (Varian Inc., Palo Alto, CA). Deuterated chloroform was used to dissolve the samples and chemical shifts were calibrated with respect to the chloroform peak ( $\delta = 7.26$  ppm). The surface chemistry of the polymer libraries was characterized using high throughput FTIR spectroscopy (Nicolet 6700 FTIR spectrometer, Thermo Fisher Scientific) which was carried out in an automated fashion with the use of a programmable mapping software (Atlas). Scanning electron microscopy (SEM) was used to determine size of the polyanhydride nanoparticles.

## Year 2



**Figure 1.** Western blots of the recombinant H5.

H5-M. The other is a trimer form, which was fused with a GCN4pII trimerization module to stabilize the native trimeric structure of H5 protein. The trimer is referred to as H5-T.

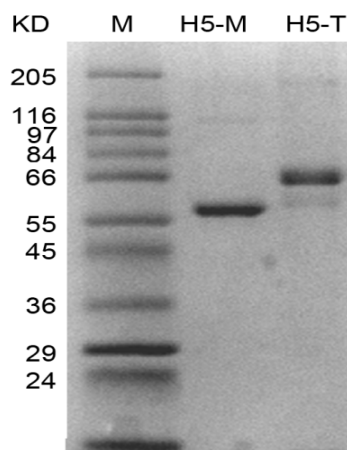
### Making recombinant baculovirus.

Recombinant baculoviruses containing the H5 genes were generated by transfecting insect SF21 cells with bacmid DNA. The baculovirus stocks were amplified for three rounds and the p3 stocks of the recombinant baculovirus were titered and used to produce the H5 proteins.

In support of the milestone related to synthesis and purification of H5 hemagglutinin (HA) antigen, we expressed the H5 HA0 ectodomain by baculovirus. The following specific steps were carried out and the results obtained are discussed.

### Construction of H5 expression vectors (bacmids)

The HA0 ectodomain of HA gene from H5N1 influenza virus (A/Whooper Swan/244/Mongolia/ 05 (clade 2.2) was cloned and expressed using a baculovirus expression system. A his-tag was added to the C-terminus of the H5 ectodomain to facilitate the purification procedure. Two forms of H5 proteins were expressed. One is a monomer form, which is referred to as



**Figure 2.** SDS-PAGE of the purified recombinant H5 proteins.

### Expression and purification of H5 proteins

The H5 monomer protein (H5-M) and trimer protein (H5-T) were expressed by infecting the SF21 cell with H5-M or H5-T baculovirus stocks. The supernatant was collected 96 h after infection, and dialyzed to get rid of the EDTA from medium. HA proteins were affinity purified by incubating supernatant overnight at 4°C with nickel-agarose beads. Non-specific nickel-bead binding was eliminated by washing with 25 mM imidazole and recombinant protein eluted from the beads with a 300 mM imidazole solution.

### Analysis of the H5 proteins

Purified proteins were separated on a 4-10% SDS-PAGE and analyzed by Western blot using anti-H5 (Vietnam 03/04) or anti-6xHis monoclonal antibody. The concentration of the purified protein was measure by the Bradford assay.

The results show that both the expressed proteins (H5-M and H5-T) were recognized by anti-H5 (Vietnam 03/04) antibody and anti-his tag antibody (**Figure 1**). The purity of the Ni-beads purified protein was >90% (**Figure 2**). The protein yield for the H5-M monomer is 2 mg/L, and the yield for the H5-T trimer is 5mg/L.

In support of the milestone related to the screening of BPNs for immune cell activations, we synthesized libraries of biodegradable polyanhydride nanoparticles of the CPH:SA system as described previously and screened them at high throughput for interactions with immune cells, specifically dendritic cells (DCs). The following specific steps were carried out:

### Culture and stimulation of DCs

Bone marrow derived dendritic cells (BMDCs) were prepared from bone marrow cells isolated from the femurs, tibias, humerus' and iliums of C57BL/6 mice (obtained from Harlan Sprague Dawley and housed within the ISU Laboratory Animal Resource Facility, Ames, IA) as previously described (1). Following mouse euthanization and bone excision, attached tissue and muscle were dissected away. The ends of the bone were cut and the marrow was flushed out using a syringe fitted with a 30 gauge needle. Each bone as flushed with 5 mL of RPMI medium containing 1% pen/strep. Large particulates were allowed to settle and then removed. After centrifugation, the cells were resuspended in DC medium (RPMI containing 1% L-glutamine, 1% penicillin-streptomycin solution, 2% HEPES, 0.5% gentamicin, 0.1% β-mercaptoethanol, and 10% heat inactivated fetal bovine serum (FBS) supplemented with GM-CSF (10 ng/mL). The cells were then placed in T75 cell culture flasks in 10 mL of DC medium containing 10 ng/mL GMCSF and incubated at 37 °C under 5% CO<sub>2</sub> atmosphere. On day 3, 10 mL of fresh DC medium with 10 ng/mL GMCSF was added. On day 6, 10 mL of the culture medium was harvested and placed in a 15 mL centrifuge tube. After centrifugation, the supernatant was decanted and the cells were resuspended into 10 mL of fresh DC medium containing 10 ng/mL GMCSF and re-inoculated into the original flask. On day 8, DCs were removed from the flasks, counted, resuspended in fresh DC medium, and transferred to 24-well plates (1 x 10<sup>6</sup> cells/well). On day 9, a portion of the cells were stained for CD11c to determine the percentage of DCs. On the same day the remaining DCs were incubated with the different stimulation treatments (films

or nanospheres). The DCs were treated with CPH:SA copolymer films and nanospheres of the following chemistries: poly(SA), 13:87 CPH:SA, 25:75 CPH:SA, 37:63 CPH:SA, 50:50 CPH:SA, and 63:37 CPH:SA. Polyanhydride nanospheres were incubated at a concentration of 0.125 mg/mL (2 cm<sup>2</sup> cell growth area). Non-stimulated (NS) DCs and LPS (200 ng/mL) treated DCs were used as negative and positive controls, respectively. After the addition of the stimulants, the DC cultures were incubated for an additional 48 h (37°C, 5% CO<sub>2</sub>) at which time the supernatants and DCs were harvested for cytokine production and cell surface marker expression, respectively. Cell viability was assessed by measuring trypan blue exclusion using a hemocytometer and light microscope.

#### Cell surface marker expression

The expression of cell surface markers including CD11c, CD86, CD40, MHC II, and CD209 were assessed after the 48 h incubation period of the DCs with the stimulation treatments. The adherent DCs were harvested from the culture dishes with vigorous pipetting, placed in polystyrene tubes (BD FALCON<sup>TM</sup>, Franklin Lakes, NJ), centrifuged (250 rcf, 10 min, 4°C), and resuspended in Fc blocking solution consisting of PBS buffer with 0.1% anti-CD16/CD32, 0.1% unlabeled hamster IgG, 1% rat IgG, 1% mouse serum, 0.1% sodium azide, and 1% FBS. After blocking, the DCs were stained and fixed for evaluation of cell surface markers using monoclonal antibodies against CD11c, MHCII, CD86, CD40, and CD209. The samples were analyzed using a Becton-Dickinson FACSCanto flow cytometer (San Jose, CA) and FlowJo (TreeStar Inc, Ashland, OR).

#### Cytokine secretion

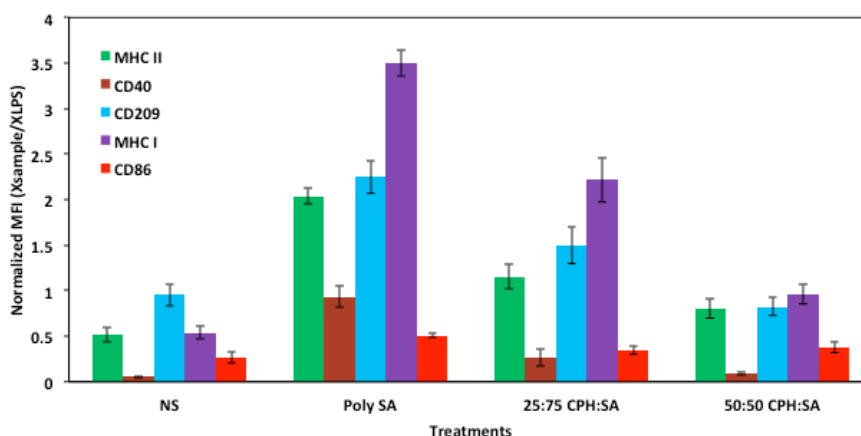
Following incubation of the DCs with the stimulation treatments for 48 h, 200 µL of the supernatants were collected and assayed for the presence of IL-6, IL-10, and IL-12-p40 using the Luminex<sup>®</sup> Multiplex assay system (Austin, TX).

#### Statistical Analysis

All data was statistically analyzed by using a one-way model ANOVA with the statistical software JMP<sup>®</sup> 7 (Cary, NC). Comparisons between treatments were made with Tukey's HSD to determine statistical significance, and p-values of less than or equal to 0.05 were considered significant. All data was log transformed for use of the one-way model ANOVA.

To assess activation of CD11c<sup>+</sup> DCs by the CPH:SA nanoparticle libraries, flow cytometry was used to measure levels of cell surface expression of co-stimulatory molecules CD86 and CD40, integrin CD209, and major histocompatibility complex molecule MHC II. Supernatants were also collected and analyzed for cytokine production, including IL-10, IL-12p40, and IL-6. LPS was used as a positive control and a non-stimulated group with medium only acted as a negative control. Prior to use in cell stimulation assays, the BMDC cultures were shown to be >90% positive for CD11c (data not shown).

The results from the analysis of cell surface marker expression following the 48 h stimulation period suggest differential regulation of the selected markers depending on the polymer



**Figure 3.** Analysis of DC activation markers upon treatment with nanoparticles by flow cytometry. Data is presented as MFI of sample normalized by that of LPS (positive control). Data is representative of a minimum of 3 replicates per stimulation group. Errors bars indicate standard error.

chemistry and the relative hydrophobicity of each formulation as shown in **Figure 3**. The SA-rich (least hydrophobic) chemistries demonstrated the ability to best promote the expression of MHC I, MHC II, CD86, CD40, and CD209. The chemistry-dependent up-regulation of the cell surface markers in comparison to the non-stimulated control is clearly observed with SA-rich nanoparticle compositions. Enhanced expression of these markers clearly correlates with the amount of SA in the nanoparticles, exhibiting a maximum for the poly (SA) nanoparticles. In addition to the MFI measurements, measurements of cells as percent positive for each of the specific markers was performed, and the results were consistent with the observed MFI for each specific cell surface marker (data not shown).

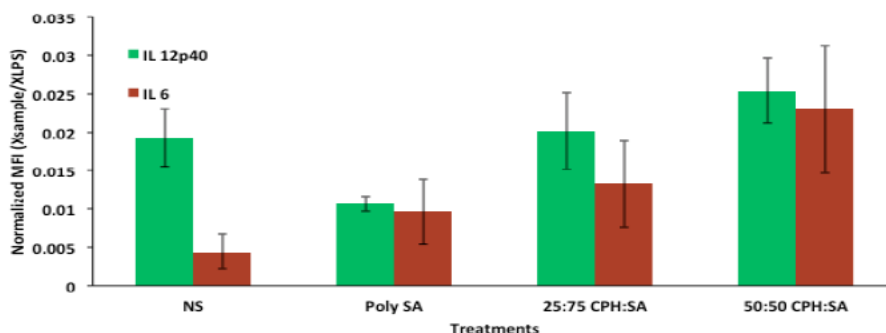
Supernatants were collected and assessed for cytokine concentrations. The results (**Figure 4**) suggest differential cytokine production (enhancement of IL-6 and regulation of IL-12p40) in response to the composition of CPH:SA nanoparticles. However, the trend for cytokine production is counter to the trend observed with the cell surface marker expression. The more hydrophobic or CPH-rich chemistries appear to better promote production of IL-6 and IL-12p40 as shown in Figure 4. IL-10 was not produced by any of the treatment groups, so the data is not included in the figures or histograms.

To synthesize HA-loaded polyanhydride nanoparticles, the following specific steps were carried out:

#### Hemagglutinin-loaded Nanoparticle Synthesis.

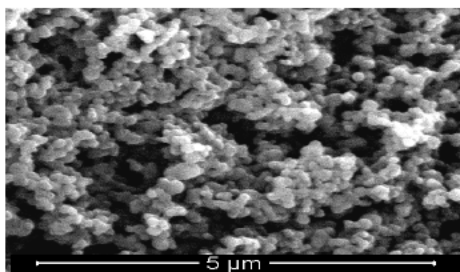
Polyanhydride nanoparticles loaded with two percent HA protein were synthesized with varying molar ratios of sebacic acid (SA), 1,6-bis(p-carboxyphenoxy)hexane (CPH), and 1,8 bis(p-carboxyphenoxy)-3,6-dioxaoctance (CPTEG). Three copolymers were used for particle fabrication: 20:80 CPH:SA, 20:80 CPTEG:CPH, and 50:50 CPTEG:CPH. Briefly, the polymer was dissolved in chilled methylene chloride at a concentration of 20 mg/mL with 2% HA protein. The solution was sonicated for approximately 30 seconds, and then poured into pentane

chilled with a liquid nitrogen bath at a ratio of 1:200 solvent: non-solvent. Protein-loaded particles were then collected via vacuum filtration and characterized by scanning electron microscopy. As shown in **Figure 5**, scanning electron microscopy images show the size and morphology of HA-loaded nanoparticles to be consistent with previous work in polyanhydride nanoparticles with other proteins.



**Figure 4.** Analysis of cytokine secretion upon treatment with nanoparticles. Data is presented as concentration of sample normalized by that of LPS (positive control). Data is representative of a minimum of 3 replicates per stimulation group. Errors bars indicate standard error.

#### Stability and Release of HA from Polyanhydride Nanoparticles



**Figure 5.** Polyanhydride nanoparticles loaded with 2% HA.

To study the stability and release of HA from polyanhydride nanoparticles, a new release method is being designed. In previous work, protein release via the erosion of polyanhydride particles was observed in 0.5 mL microcentrifuge tubes (2). Protein-loaded particles were incubated in phosphate buffered saline (PBS) with 0.01% w/v sodium azide at 37°C. Samples of the released protein were then taken periodically by completely removing solution and replacing with fresh PBS.

One shortcoming of this microcentrifuge tube method involves the build-up of polymer degradation products within the buffer between sampling. These degradation products are acidic and the decrease in pH can cause instabilities within many proteins. One solution is to incubate the particles in dialysis cassettes, rather than centrifuge tubes. These dialysis cassettes allow the removal of acidic degradation products and the maintenance of a balanced pH, better mimicking the body's constant motion of fluid in vivo. However, particles within a dialysis cassette tend to sink to the bottom, despite being well dispersed initially. Degradation products as well as protein can become trapped among the settled particles still leading to acidic microenvironments.

We have designed a new release method using 1 mL microcentrifuge tubes with dialysis membranes incorporated into specially designed caps (GE Healthcare, Piscataway, NJ). The release from HA-loaded particles takes place in 250  $\mu$ L of PBS with 0.01% w/v sodium azide loaded into the dialysis tube. This tube was then placed in a 50 mL conical centrifuge tube with

additional buffer and then placed on a rocker to flip end over end. This motion allows the constant dispersion of particles as well as allowing dialysis to take place, best mimicking the *in vivo* environment.

### Protein Quantification

A colorimetric micro bicinchonic acid (microBCA) assay (Pierce, Rockford, IL) was used to identify the concentration of HA protein released from polyanhydride particles. Although many researchers use the absorbance of bovine serum albumin (BSA) or ovalbumin (OVA) to create standard curves, the most accurate results are obtained by using the protein of interest, HA, to create standard curves. However, the HA standard curve has a relatively low slope, which can create misleading results as small changes in the absorbance measured can correlate to large changes in protein concentration. Therefore, an appropriate assay for HA quantification was optimized through the trials of several other colorimetric techniques (i.e., Bradford assay) as well as fluorescence-based techniques.

### Enzyme-linked Immunosorbant Assay (ELISA)

An enzyme-linked immunosorbant assay (ELISA) has been optimized to study the antigenicity of released HA protein. In this case, if the stability of the protein has been preserved then the ability of anti-HA antibodies to bind the protein should be preserved. Briefly, the optimized conditions begin with coating a high-binding 96-well plate with 100  $\mu\text{L}$ /well of released HA protein diluted to 5  $\mu\text{g}/\text{mL}$  and 0.5  $\mu\text{g}/\text{mL}$  in phosphate buffer saline (PBS). After incubating the plate at 4°C overnight, the wells are emptied and filled with 300  $\mu\text{L}$ /well blocking solution of PBS with 0.05% Tween 20 (PBS-T) and 1% BSA for 2 h at room temperature. The plate is then washed with PBS-T and incubated with 100  $\mu\text{L}$ /well anti-HA (BEI Resources NR 2731) diluted 1:1000 in PBS-T at 4°C overnight. The following day the wells are washed with PBS-T and incubated with 100  $\mu\text{L}$ /well secondary antibody, alkaline phosphatase conjugated anti-mouse IgG(H+L) (Jackson ImmunoResearch, West Grove, PA), diluted 1:1000 in PBS-T for 2 h at room temperature. Finally, following an additional wash step with PBS-T, 1 mg/mL phosphate substrate (Sigma) is prepared in a substrate buffer of sodium carbonate and magnesium chloride. After adding 100  $\mu\text{L}$ /well of the substrate to each well, the developing plate is read by a microplate spectrophotometer at 405 nm.

### Western Blot

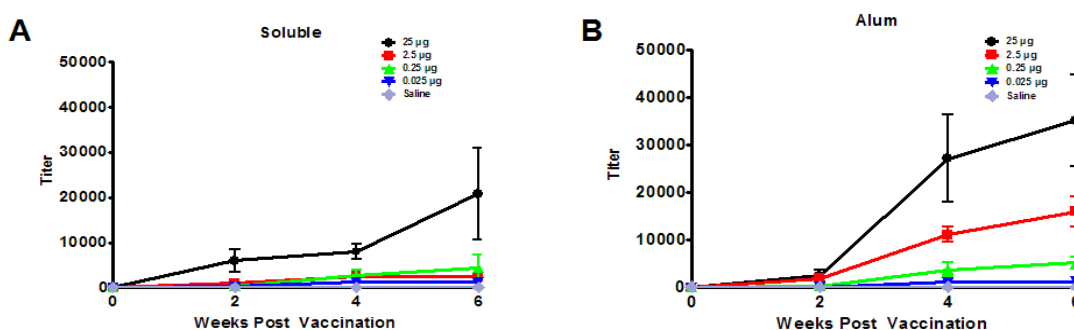
We optimized western blot analysis by performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis for 1 h at 150V to separate the released protein by molecular weight. The protein was transferred from the gel to a polyvinylidene fluoride (PVDF) membrane for 1.5 h at 100V. Following transfer, the PVDF membrane was incubated in blocking solution, tris saline with tween 20 (TBS-T) with 5% milk, on a rocker overnight at 4°C. The next day, the membrane was rocked with 10 mL anti-HA (BEI Resources) diluted 1:1000 in blocking solution for 2 h at room temperature. After rinsing with TBS-T, the secondary HRP-conjugated antibody (Jackson ImmunoResearch) was diluted 1:100,000 in TBS-T and rocked with the membrane for an additional 2 h. Finally, the membrane was washed an additional time with TBS-T and developed using an ECL Plus substrate (Pierce).

Preliminary dose titration studies with H5 HA antigen. The following specific steps were carried out :



### Dose Titration

Full-length HA H5 protein was obtained from Boehringer Ingelheim Vetmedica to perform initial experiments. The protein was produced as a full-length protein and exists as monomers, dimers, and trimers. Using this protein we tested titrating dosages of the protein unadjuvanted or using traditional adjuvants via a parenteral route, subcutaneously, to examine the humoral response curve after a single dose. This was done to gain insight into the optimal dosage i.e. amount of protein that will be needed for designing a vaccine containing our novel polyanhydride nanoparticle platform against H5N1 influenza.



**Figure 6.** Total anti-H5 serum antibody response of mice.

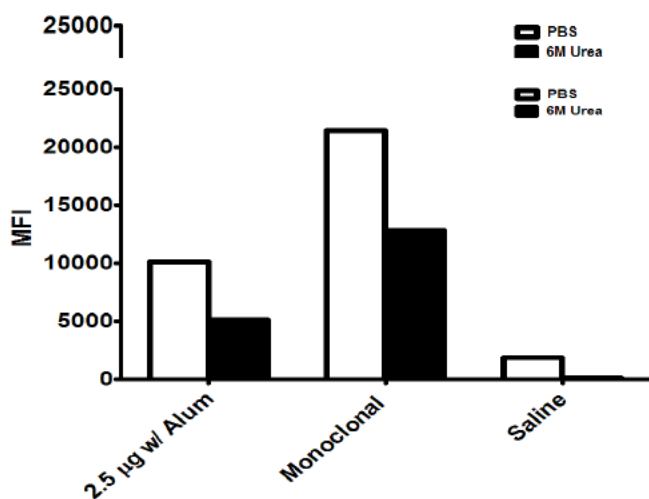
### Antibody Avidity

The dosage titration experiment has also afforded us the opportunity to optimize and design more sophisticated measures of antibody avidity. Using principles adapted from van Gageldonk et al. (3) and Stenger et al. (4), full length H5 HA was conjugated to carboxylated magnetic beads (Bio-Rad Laboratories, Hercules, CA) according to manufacturer protocols at three different antigen to bead ratios (5, 10, and 12.5 µg/1.25 x 10<sup>6</sup> beads). Three dilutions of serum from the 2.5 µg HA adjuvanted with Alum group seen above, a saline vaccinated negative control, and of a H5 monoclonal antibody NR-2743 (BEI Resources) were incubated 1:1 with conjugated beads prepared from the ratios described previously so that there were 4000 beads per well in a 96 well Bio-Plex Pro Flat-Bottom plate (Bio-Rad). The serum/bead mixture was incubated in the dark for 2 hours at room temperature (RT). The beads were washed three times using a Bio-Plex Pro II magnetic bead washer (Bio-Rad). A biotinylated secondary mouse IgG (eBioscience San Diego, CA) was diluted to 5 µg/mL in buffer and 50 µL was added to the beads. The plate was incubated for 1 h at room temperature. The plate was washed three times and finally 50 µL of a 10 µg/mL Streptavidin-Phycoerythrin (eBioscience) solution was added. Plates were incubated at RT for 30 min, washed, and subsequently mean fluorescence intensities (MFIs) were captured using the Bio-Plex 200 system (Bio-Rad). No appreciable differences in MFIs were indicated between the three formulations of conjugated beads. Therefore we proceeded to adapt the antibody avidity protocol using 5 µg conjugated beads.

The preparation protocol and initial incubation steps remained the same from the above protocol described. After the initial antibody/bead incubation step a chaotropic reagent step is added to dissociate antigen/antibody interactions to measure the strength of those reactions. Urea dissociation was more consistent in the Stenger et al protocol so we optimized those conditions

for our assay. Three different concentrations of urea were prepared (3, 6, and 9M) and a PBS control. After washing the beads, 50  $\mu$ L of the urea solutions or control were added to the wells and incubated for 15 min at RT. The beads were washed and the subsequent secondary steps were performed and MFIs measured. 6M urea proved to be the optimal concentration of chaotropic reagent as it consistently resulted in a reduction of 30-70% MFI from the PBS control.

**Figure 6A** shows the total anti-H5 serum antibody response of mice vaccinated with soluble protein subcutaneously over a six-week time frame. 25  $\mu$ g soluble protein only doses elicited measurable titers from baseline levels. 2.5  $\mu$ g and lower soluble doses remain at relatively



**Figure 7.** MFIs from test sera and monoclonal antibody for avidity. Avidity Index reported as  $(\text{MFI urea treated})/(\text{MFI untreated}) * 100\%$

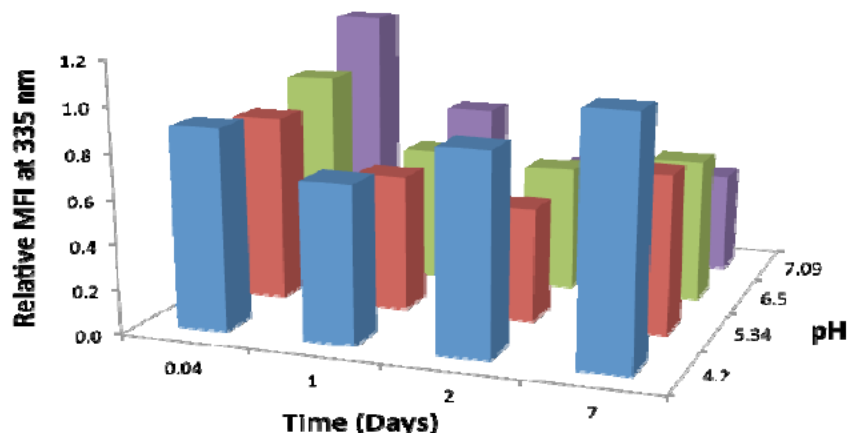
baseline levels. When the full length HA protein is adsorbed to alum we are able to detect measurable serum immunoglobulin titer differences in the 25  $\mu$ g and 2.5  $\mu$ g groups (**Figure 6B**). The antigen dosage titration has provided great insight into the dosage of protein to begin designing vaccine using the novel polyanhydride nanoparticle platform.

**Figure 7** is representative histogram of the MFIs obtained from test serum and the monoclonal antibody used. An avidity index can then be calculated using the PBS control and urea treated MFI value where the MFI obtained in the presence of urea is divided by the MFI obtained in the absence of urea (PBS) and multiplied by 100%. The

results indicate an avidity index of 50.70 for the 2.5  $\mu$ g alum group, a 59.62 avidity index for the monoclonal antibody, and 4.71 for the saline negative control.

### Year 3

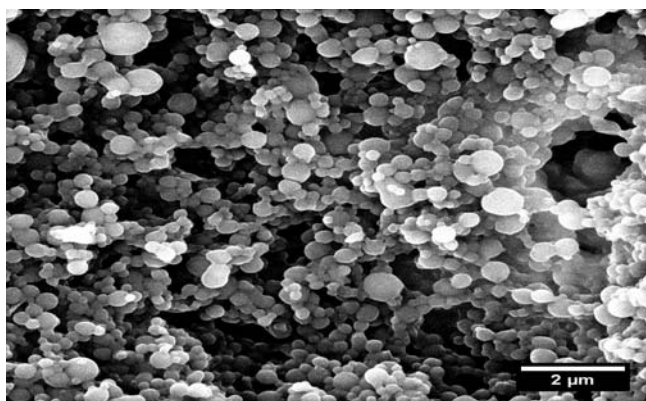
To rationally determine the optimal polyanhydride chemistries to release stable hemagglutinin



**Figure 8.** Tertiary structure of HA shows increased stability under acidic pH. Mean fluorescence intensity (MFI) of the HA maximum peak (335 nm) show increased stability under acidic conditions. HA incubated at neutral pH shows deterioration of the protein's tertiary structure.

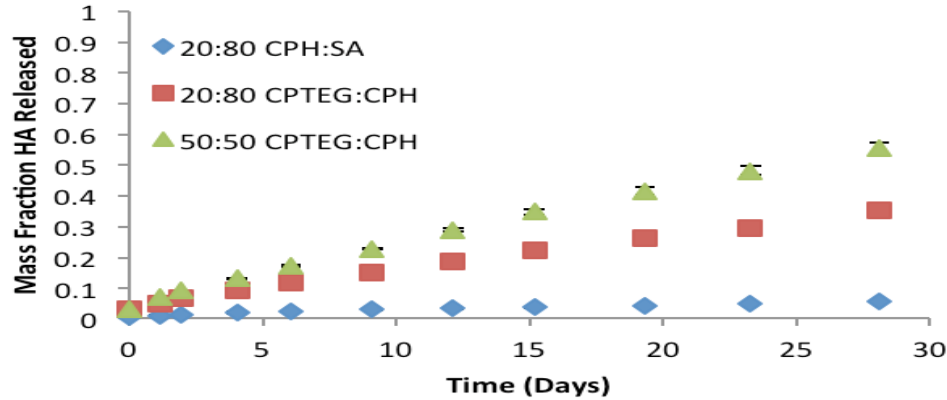
(HA) a full-length HA H5 protein (Boehringer Ingelheim Vetmedica) was incubated in phosphate buffered saline (PBS) with varying pH and temperature. By studying the tertiary structure of HA over a period of one week it was shown the protein is more stable in mild acidic conditions versus normal pH (**Figure 8**).

Polyanhydride nanoparticles containing two percent HA were synthesized via a solid/oil/oil method to determine protein stability and release kinetics from nanoparticles. Three polyanhydride copolymers were chosen based on the pH of their degradation products (4.5-5.5) to stabilize HA: 20:80 CPH:SA, 20:80 CPTEG:CPH, and 50:50 CPTEG:CPH. Briefly, the polymer was dissolved in chilled methylene chloride at a concentration of 20 mg/mL with 2% HA protein. The solution was sonicated for approximately 30 s and = poured into pentane chilled with liquid nitrogen at a solvent:non-solvent ratio of 1:200. Protein-loaded particles were collected via vacuum filtration and characterized by scanning electron microscopy (**Figure 9**).



**Figure 9.** SEM of 2% HA nanoparticles. Scanning electron microscopy image displaying the approximate size and morphology of 2% HA 20:80 CPH:SA

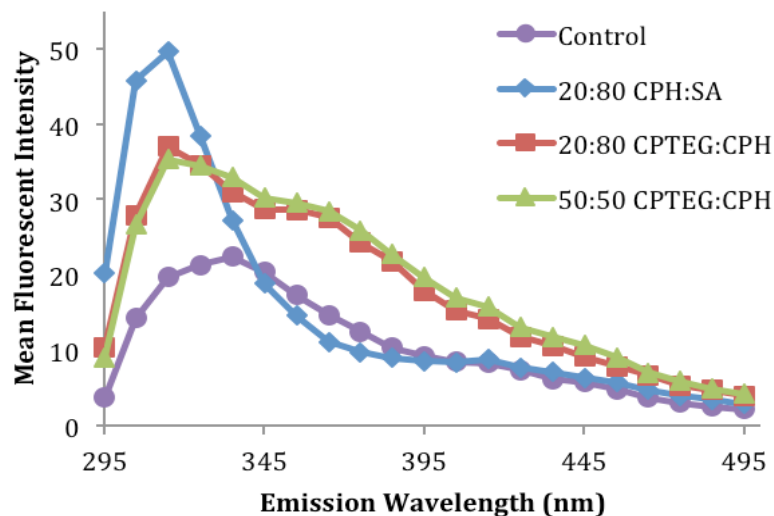
The nanoparticles were incubated in PBS and sampled for protein release for approximately one month. Encapsulation of HA into polyanhydride particles was found to sustain release of the protein with zero order kinetics (**Figure 10**), consistent with our previous work utilizing other proteins. Structural studies also shown the primary (SDS PAGE) and tertiary structure (fluorescent spectroscopy) structure of the protein was maintained upon release. The tertiary structure of released protein showed an increase in mean fluorescent intensity, which may indicate the exposure of buried fluorescent residues during conformational changes upon release (**Figure 11**). Noting that the pH of polyanhydrides corresponds to the conformational change of HA, shifts in the peak emission wavelength show conformational changes and increased stability of released HA consistent with previous experiments.



**Figure 10.** Release kinetics of HA. Polyanhydride nanoparticles showed zero order release kinetics of HA over approximately one month. As expected, more amphiphilic copolymers (CPTEG:CPH) released protein faster than more hydrophobic copolymers (CPH:SA).

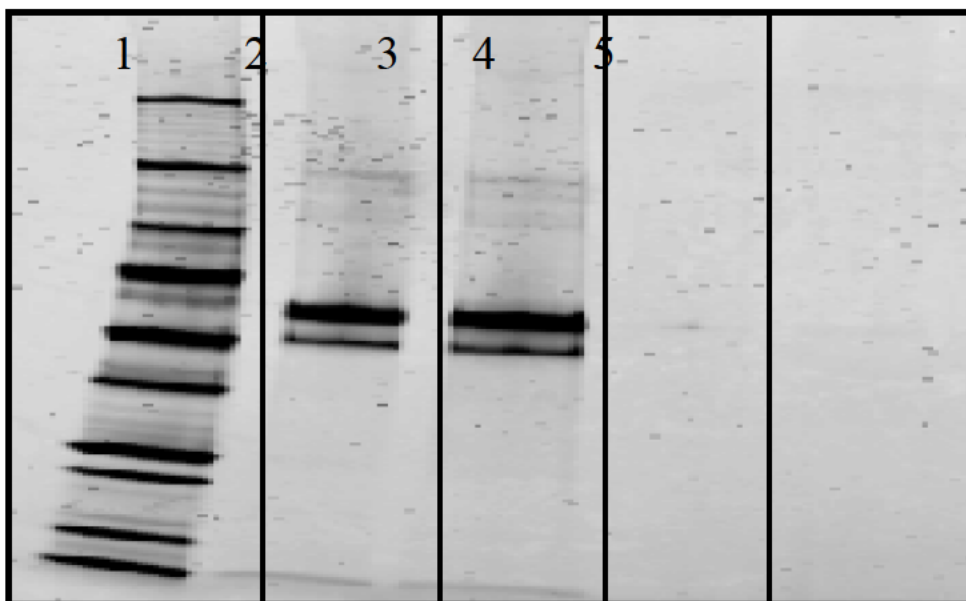
With the future intention of observing differences between HA monomer (H5-M) and trimer (H5-T) immunogenicity, as well as cross-protection between H5N1 influenza clades, we sought to observe the stability of a clade 2.2 H5 HA A/Mongolia/Whooper Swan/244/05 protein synthesized in both monomer and trimer forms. This protein was synthesized by Dr. Wuwei Wu in the laboratories of Dr. Susan Carpenter at ISU.

*In vitro* work with the recombinant H5 HA A/Mongolia/Whooper Swan/244/05 identified sensitivities of the protein to different buffers as well as processes previously employed for dry powder fabrication into polyanhydride particles. Low solubility due to aggregation and precipitation led to no or minimal detection of the protein in both quantification and structural assays. The solubility of the protein was improved by increasing the pH of buffer (pH 8) further away from the protein's isoelectric point. To improve detection in quantification assays, it was found that a combination of 2% sodium dodecyl sulfate (SDS) and 25 mM sodium hydroxide (NaOH) with HA monomer elicited the most accurate results using a microBCA assay.



**Figure 11.** Tertiary structure of released HA. Fluorescent spectroscopy shows the conformational changes and increased stability of HA tertiary structure upon release from polyanhydride nanoparticles.

It was also determined that the lyophilization process led to the instability of the protein. Both the stabilized H5-T and H5-M lose immunogenicity and function when lyophilized to a dry powder form. Soluble HA (sH5), or protein that was never lyophilized, was determined to have increased stability over reconstituted protein. Soluble HA was easily quantified with a microBCA assay, and improved detection was observed in ELISA (antigenicity), SDS PAGE (primary structure), circular dichroism (secondary structure), and fluorescent spectroscopy (tertiary structure) experiments. For example, in **Figure 12**, lanes loaded with equivalent amounts of protein showed improved detection with soluble HA versus reconstituted HA, even in the presence of additives to increase protein solubility.



**Figure 12.** Soluble HA shows increased detection over reconstituted protein in SDS PAGE. SDS PAGE lanes loaded with equivalent amounts of HA protein exhibited darker bands with soluble protein versus reconstituted, even with additives such as SDS and NaOH to increase solubility. Lane assignments: 1) Molecular weight ladder 2) Soluble HA 3) Soluble HA + SDS/NaOH 4) Reconstituted HA and 5) Reconstituted HA + SDS/NaOH.

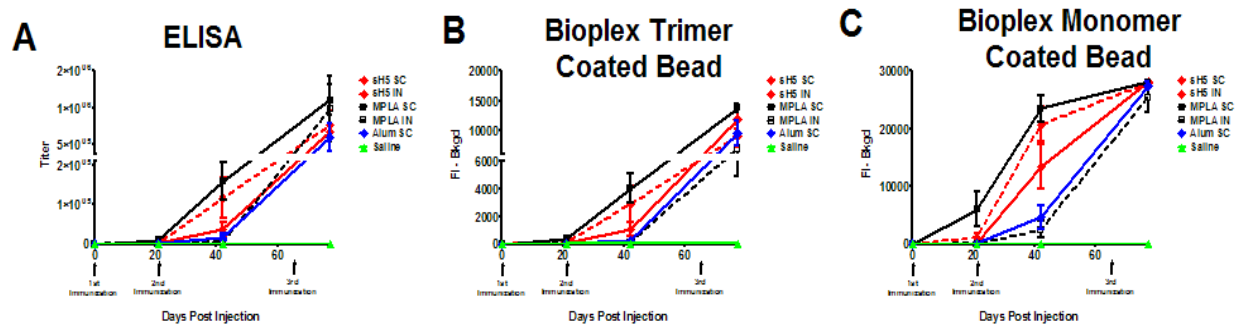
Although solid/oil/oil nanoprecipitation produced higher encapsulation efficiencies, the fabrication of polyanhydride nanoparticles was modified to a water/oil/oil (w/o/o) method to accommodate encapsulating protein in the more stable, soluble form. Briefly, 500  $\mu\text{g}$  of HA dissolved in water was added to 2.5 mL of methylene chloride containing 50 mg of polymer. The solution was homogenized for 60 seconds and poured into 625 mL pentane. The precipitated nanoparticles were collected via vacuum filtration. Studies are ongoing to determine the encapsulation efficiency of HA in polyanhydride nanoparticles and the stability of HA under particle fabrication conditions.

In parallel to the *in vitro* stability studies, we also performed *in vivo* experiments with the recombinant HA proteins. Our previous work showed that a 10  $\mu\text{g}$  dose of recombinant HA protein was optimal. We also designed a sensitive antibody quantitation platform using Bioplex based methods and technology that allowed for the simultaneous examination of antibody reactivity to both the stabilized trimer and monomer forms of the H5 antigen. This new method enabled the minimization of serum sample and protein usage and resulted in throughput enhancement.

Due to the instability of lyophilized HA, soluble forms of the H5-T were used to immunize BALB/c mice subcutaneously and intranasally (10  $\mu\text{g}$  dose) with and without different traditional adjuvants: monophosphoryl lipid A (MPLA) or aluminum salts (Alum) (**Table 1**). These studies will be used to benchmark future work with nanoparticle-based adjuvants.

**Table 1. Immunization Groups of Soluble H5-T**

Group	Antigen	Adjuvant	Route
sH5 SC	10 µg H5-T in PBS	None	Subcutaneous
sH5 IN	10 µg H5-T in PBS	None	Intranasal
MPLA SC	10 µg H5-T in PBS	10 µg MPLA	Subcutaneous
MPLA IN	10 µg H5-T in PBS	10 µg MPLA	Intranasal
Alum SC	10 µg H5-T in PBS	Alum	Subcutaneous
Saline	None	None	Subcutaneous



**Figure 13.** Serum antibody response of mice immunized with soluble H5-T. BALB/c mice immunized with soluble doses of H5-T show increasing amounts of antigen specific antibody after boost.

Weak titers via ELISA or Bioplex antibody quantitation were observed 21 days post a single immunization (Figure 13A-C). A boost immunization was administered at the 21 day post immunization (DPI) timepoint. Serum samples obtained at 42 days post immunization showed increasing amounts of antigen-specific antibody at this time point.

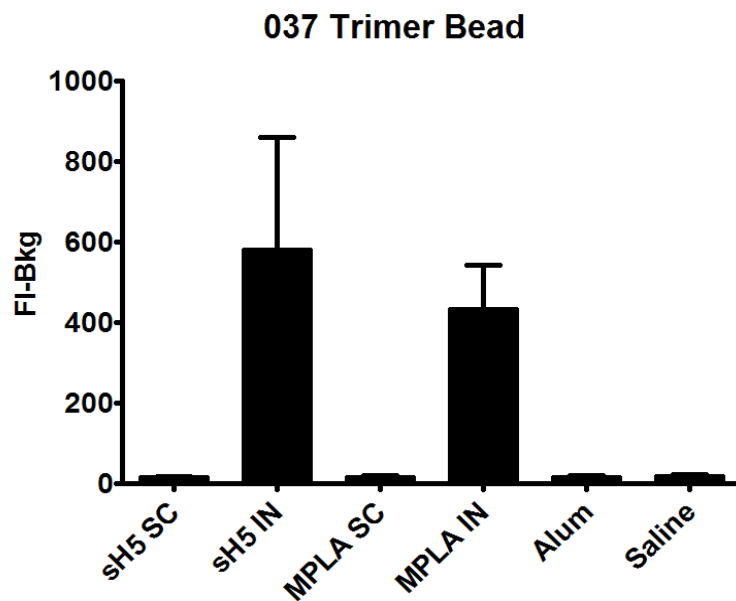
Neutralizing activity against an equine infectious anemia virus expressing the H5 HA A/Mongolia/Whooper Swan/244/05 was observed at the 42 DPI time point, once again pointing to the importance of using soluble protein for vaccine preparations. This was evidenced by the observation of negligible antibody neutralizing activity until this point (**Table 2**).

**Table 2. Neutralization Antibody Titer**

Group	Neutralization Titer	
	21 DPI	42 DPI
sH5 SC	<50 ± 0	484.78 ± 284.35
sH5 IN	<50 ± 0	2203.08 ± 1495.09
MPLA SC	<50 ± 0	5784.01 ± 2523.37
MPLA IN	<50 ± 0	22.90 ± 21.90
Alum SC	<50 ± 0	13.06 ± 12.06
Saline	<50 ± 0	<50



The highest amount of neutralizing activity was observed in the groups receiving a Toll like receptor (TLR) agonist as an adjuvant. Alum did not show effective adjuvanticity in eliciting neutralizing titer, although all whole protein titers were relatively similar. Neutralization titer data for the 77 DPI time point is currently being evaluated. The stability of the protein appears to be of paramount importance in these studies as the acidity due to Alum or crosslinking adsorption may have led to degradation of the neutralizing epitopes of HA. This study also points to the importance of using a prime-boost strategy for eliciting neutralizing antibody from these immunization preparations. As expected, only intranasal immunizations were able to elicit antigen specific IgA antibody in the bronchio-alveolar lavage fluid at the end of the study (Figure 14).



**Figure 14.** Antigen specific IgA. Intranasal immunizations of soluble HA elicit antigen specific IgA antibody in bronchioalveolar lavage fluid.

Upon optimization of the w/o/o process, the next step was to study the structural and antigenic stability of H5-T and H5-M released from nanoparticles. To complete these studies, a fetuin assay was being developed to test the immunological function of H5-T and H5-M upon release. Fetuin protein, representing the sialic acid residues that bind HA in the lung, was coated on a high binding, 96-well plate at 1 µg/mL and incubated overnight at 4°C. The following day, each well was blocked with 300 µL of phosphate buffered saline with 0.05% Tween 20 (PBS-T) and 1% gelatin. After blocking for two hours at room temperature, the plate was washed with PBS-T and incubated with a monoclonal antibody overnight at 4°C. Finally, results were determined by alkaline phosphatase absorbance at 405 nm.

The efficacy of single and multiple dose polyanhydride particle-based H5 HA vaccines were planned. The previous data with subcutaneous immunizations of HA protein has led us to initiate studies examining immune responses from subcutaneous immunizations of HA protein encapsulated including encapsulated ligands of TLR's (Poly I:C) and inflammasome (Poly dA:dT) receptors. Previous *in vitro* data with encapsulated Poly I:C showed increased inflammatory cytokine secretion from bone marrow derived macrophages that was dependent on



phagocytic action. The immunization groups for initial polyanhydride nanoparticle-based immunizations are indicated in **Table 3**. A parallel study examining the efficacy of intranasal immunizations were performed with the same groups as shown in **Table 3**.

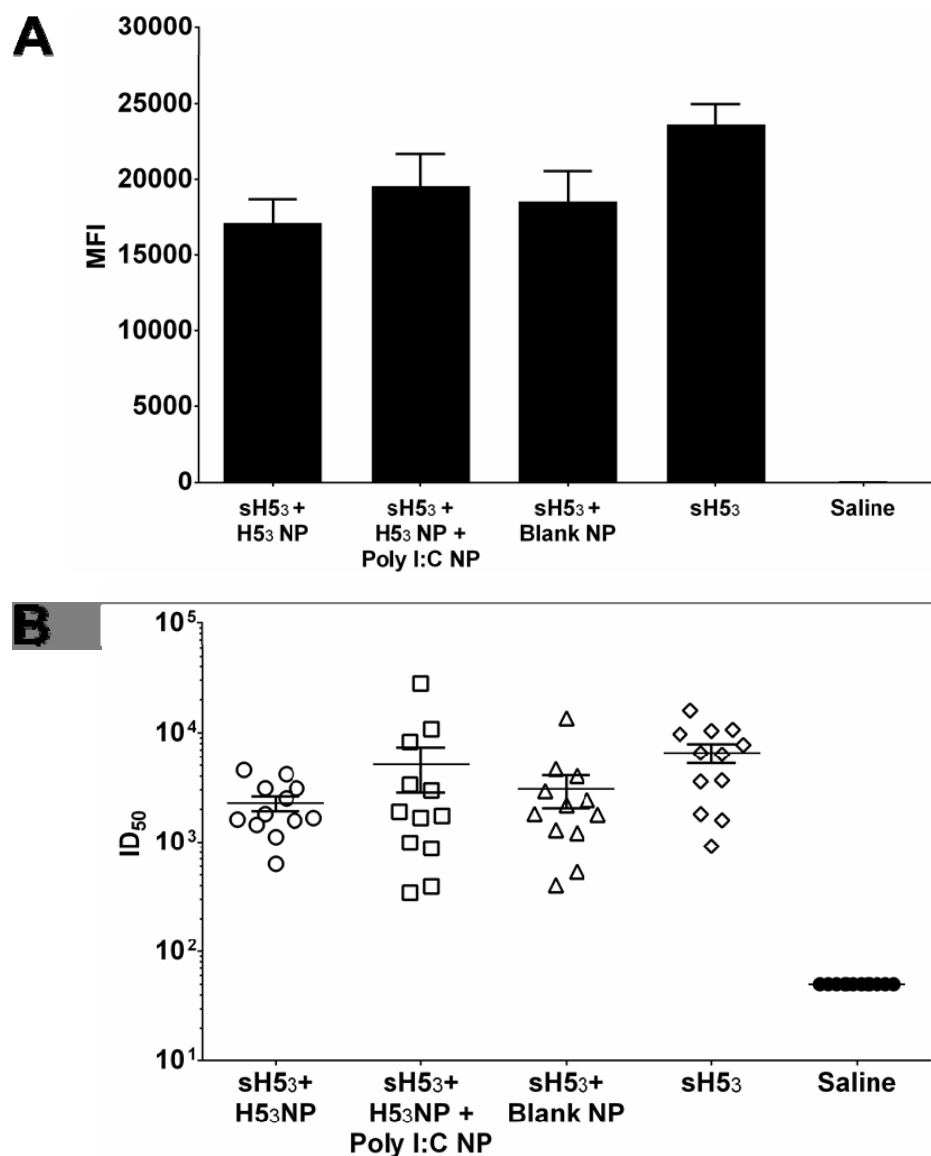
**Table 3. Immunization Groups for Polyanhydride Nanoparticles**

<b>Immunization Group</b>	<b>Abbreviation</b>	<b>Second Immunization</b>
SC-7.5 µg free + 2.5 µg 20:80 CPTEG:CPH	20:80 C:C	Day 21 post injection (p.i.)
SC-7.5 µg free + 2.5 µg 20:80 CPTEG:CPH + 5 µg Poly I:C 20:80 CPTEG:CPH	20:80 C:C <sub>poly I:C</sub>	Day 21 p.i.
SC-7.5 µg free + 5 µg Poly I:C + 2.5 µg 20:80 CPTEG:CPH	20:80 C:C + sPoly I:C	Day 21 p.i.
SC-7.5 µg free + 2.5 µg 20:80 CPTEG:CPH + 5 µg Poly dA:dT 50:50 CPTEG:CPH	20:80 C:C <sub>poly dA:dT</sub>	Day 21 p.i.
SC-7.5 µg free + 5 µg Poly dA:dT + 2.5 µg 20:80 CPTEG:CPH	20:80 C:C + sPoly dA:dT	Day 21 p.i.
10 µg MPLA x 2	MPLA	Day 21 p.i.
10 µg soluble H5 Trimer	sH5T	Day 21 p.i.
Saline	Saline	None

*In vitro* T cell proliferation assays were also developed to examine the ability of our polyanhydride nanoparticle immunization platform to activate macrophages and the subsequent antigen presenting capabilities of these macrophages using transgenic T cells specific for outlined peptide sequences.

#### **Year 4**

Mice were immunized with soluble H5 hemagglutinin trimer (sH5<sub>3</sub>) alone or in conjunction with H5<sub>3</sub>-loaded, poly I:C-loaded, or blank 20:80 CPTEG:CPH polyanhydride nanoparticles. All mice received a total of 10 µg H5<sub>3</sub> per immunization delivered subcutaneously, with booster immunizations at days 21 and 42 as well. At 56 days post-initial immunization, serum was collected from the animals to determine the presence of anti-H5<sub>3</sub> antibodies. A fluorescent, multiplex based assay was used to quantify the total serum anti-H5<sub>3</sub> IgG. All treatment formulations induced antibodies significantly greater than the saline control; however, there remained little variation among the formulations themselves (**Figure 15A**). H5-pseudotyped reporter viruses were also used to determine the neutralizing antibody titers. Consistent with total antibody, neutralizing antibody titers were quite similar among formulations (**Figure 15B**).



**Figure 15.** Antibody responses to H53 immunization. Mean fluorescent intensity (MFI) quantifying total IgG serum antibody via a multiplex assay (A). Neutralizing antibody titer determined via H5-pseudotyped reporter viruses (B).

To determine the ability of the total and neutralizing antibodies to provide protection, all mice were intranasally challenged with a live, low pathogenic H5N1 influenza virus 63 days post-initial immunization. Mice receiving saline immunizations began to lose weight 4 days post-challenge and continued to lose approximately 20% of their total body weight before recovering. In contrast, all mice immunized with H5<sub>3</sub> and/or polyanhydride nanoparticle formulations maintained their body weight similar to naïve, non-infected control mice. The viral load present in the lung tissue of mice was also determined 3 days post-challenge. As expected, all immunization formulations were able to induce a reduction of viral load upon challenge. Similarly, bronchoalveolar lavage fluid of immunized mice collected 3 days post-challenge

showed a large decrease of inflammatory cytokines compared to the saline control (data not shown).

These initial results display a proof-of-concept for this vaccine platform, emphasizing the strength and immunogenicity of the hemagglutinin trimer cloned and expressed. Mice receiving sH5<sub>3</sub> alone induced neutralizing antibody titers that were protective against a low pathogenic challenge, similar to mice receiving polyanhydride nanoparticles. Previous work has underlined dose sparing properties of polyanhydride nanoparticles, allowing the enhancement of immune responses towards suboptimal doses of antigen. Likewise, polyanhydride nanoparticles have displayed enhancement of cell-mediated immunity, an important aspect of vaccines targeting viral pathogens such as influenza.

## **Platform B**

### **Year 1**

In support of the design of DNA plasmid encoding reporter gene in place of influenza proteins, the DNA sequence coding for the Luciferase reporter system has been subcloned from the gWIZ-Luc plasmid and inserted into the pVAX1 backbone. After completion of the subcloning step, the pVAX1-Luc plasmid generated has been introduced into competent DH5 $\alpha$  *E. Coli* bacteria, amplified and purified. Part of the purified plasmids has been used for the DNA sequencing of the insert. The functionality of the encoded protein has also been checked through an *in vitro* transfection assay. Briefly, HEK293T cells were transfected with the pVAX1-Luc plasmid combined to Lipofectamine 2000. Proteins were then extracted and Luciferase expression was quantified using a Luciferase assay kit.

The plasmid encoding the H5N1 HA has been obtained and subcloned into the pVAX1 backbone. The plasmid was then expanded in DH5 $\alpha$  *E. Coli* bacteria and purified. Purified pVAX1-H5HA plasmid has been sequenced and shown 100% sequence homology of the insert. This constructed plasmid has been tested for its *in vitro* expression. HEK293T cells were transfected with pVAX1-H5HA plasmid with Exgene (Fermentas, GlenBurnie, MA) transfection reagent. Proteins were then extracted and purified using MagneHis Protein Purification System (Promega, Madison, WI) and expression of H5HA protein were analyzed by Western Blot. To determine the transgene expression of pVAX1-H5HA and pVAX1-Luc plasmids in tibialis anterior muscle in mouse, 10  $\mu$ g of plasmid DNA were administered with/without 0.01% w/v Pluronic block copolymer SP1017. To compare the transgene expression of pVAX1-Luc with gWIZ-Luc plasmid DNA, we also administered gWIZ-Luc plasmid DNA to one group of mice. It has been found that pVAX1-Luc vector also efficiently expressed the transgene as gWIZ-Luc in *in vivo*. pVAX1-Luc plasmid DNA formulated with 0.01% w/v SP1017 enhanced transgene expression in mice.

### **Year 2**

In order to determine the Luciferase and H5HA expression *in vivo*, 10  $\mu$ g of pVAX1-Luc (control plasmid) plasmid DNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered i.m. in tibialis anterior muscles in BALB/c mice (5 mice/group). Injection formulations of DNA and DNA with pluronics were injected in left and right tibialis anterior muscles respectively. Mice were sacrificed on day 7 after i.m. injection. Tibialis anterior muscles were harvested, weighed, snap frozen in liquid N<sub>2</sub> and stored at -80°C for isolating mRNA to do RT-PCR for determining the transgene expression of H5HA and Luciferase gene and also the

Luciferase assay. The following experiments were performed to determine the Luciferase and H5HA expression *in vivo*.

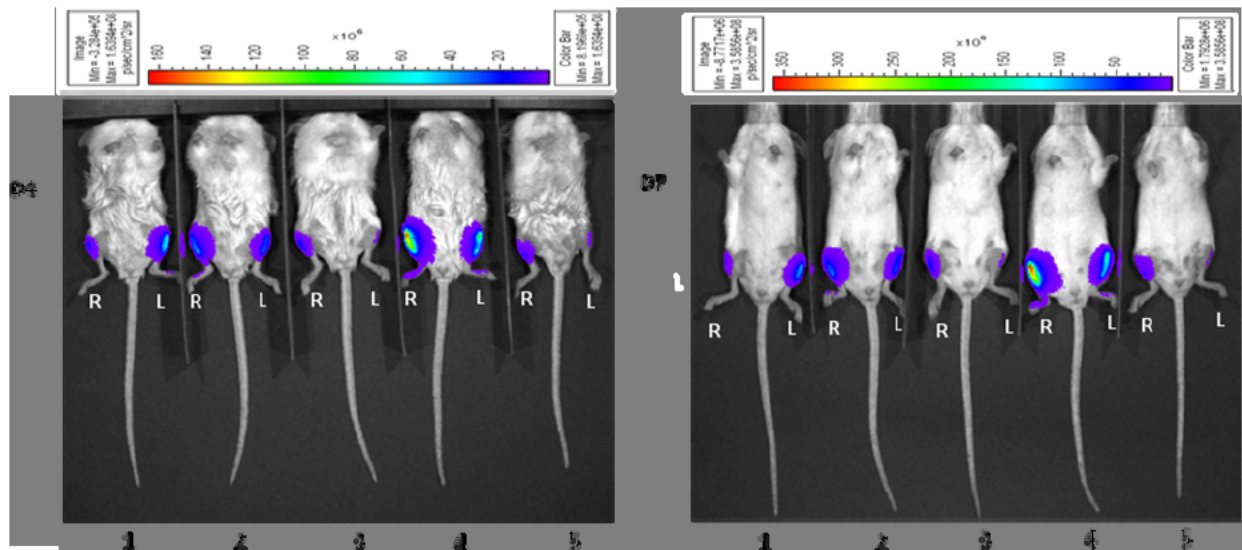
In order to determine the Luciferase expression *in vivo*, 10 µg of pVAX1-Luc (control plasmid) plasmid DNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered i.m. in tibialis anterior muscles in BALB/c mice (5 mice/group). The animals were subjected to non-invasive bioluminescence detection using the Xenogen In Vivo Imaging System-IVIS 200 on day 4 and day 7 after i.m. administration (**Figure 16** and **17**). 10 µg of naked pVAX1-Luc plasmid DNA (pDNA) produced luciferase expression in skeletal muscles in all 5 mice on day 4 and all 5 mice expressed luciferase with higher intensity on day 7 in P85 group (**Figure 16A** and **B**). But **Figure 17A** and **17B** showed that 10 µg of naked pVAX1-Luc pDNA produced luciferase expression in 4 mice out of 5 in SP1017 group. pVAX1-Luc pDNA formulated with 0.3% w/v P85 produced higher luciferase expression in all 5 mice on day 4 and day 7 compared to that of naked pDNA (**Figure 16A** and **B**). However, plasmid DNA formulated with 0.01% w/v SP1017 produced luciferase expression in 4 mice among 5 mice on day 4 and all mice expressed higher luciferase intensity compared to that of naked pDNA on day 7 (**Figure 17A** and **B**). These results are summarized in **Table 4**.

**Table 4.** *In vivo* transgene expression after a single *i.m.* administration of pDNA with/without Pluronics in BALB/c mice (5 mice/group).

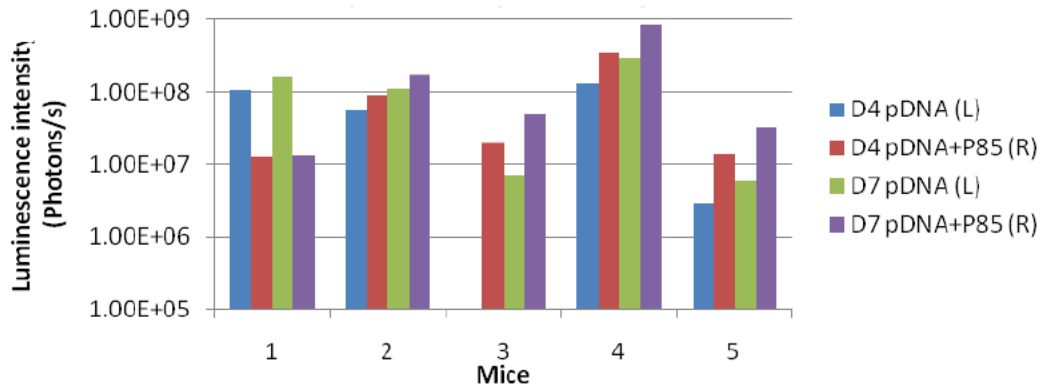
	Respondent mice (no.)	
	Day 4	Day 7
<b>P85 group</b>		
pVAX1-Luc pDNA	5/5	5/5
pVAX1-Luc pDNA+P85	5/5	5/5
<b>SP1017 group</b>		
pVAX1-Luc pDNA	4/5	5/5
pVAX1-Luc pDNA+SP1017	4/5	5/5

The reasons for variation of transgene expression could be (1) due to the technical error upon injection, could be (2) difference in transcription efficiency among animals. Tibialis anterior muscles were collected from the same animals on day 7 after single i.m. injection and subsequent *in vivo* imaging and used those muscles for analysis of the luciferase activity (**Figure 18B**). pVAX1-Luc pDNA formulated with both Pluronics P85 and SP1017 enhanced transgene expression in mice on day 4 and day 7 (**Figure 16, 17** and **18**). To compare the effects of Pluronics on transgene expression of pVAX1-Luc, the average intensity of luminescence were plotted in **Figure 12**. pDNA formulated with P85 expressed higher average luminescence intensity compared to that of SP1017 *in vivo* (**Figure 18**). **Figure 16, 17** and **18** demonstrate that both Pluronics P85 and SP1017 efficiently enhanced transgene expression of pVAX1-Luc vector in *in vivo*.

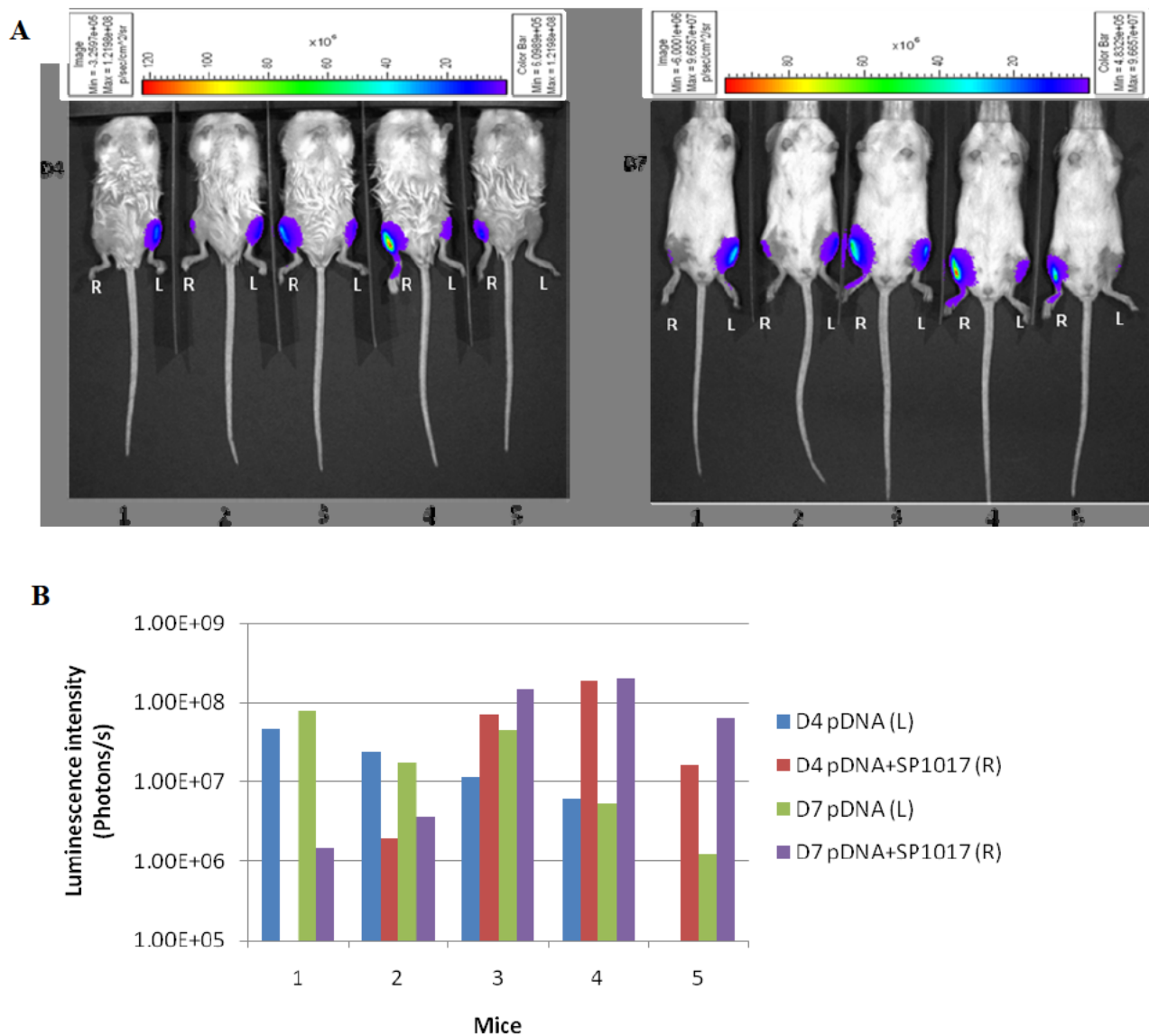
A



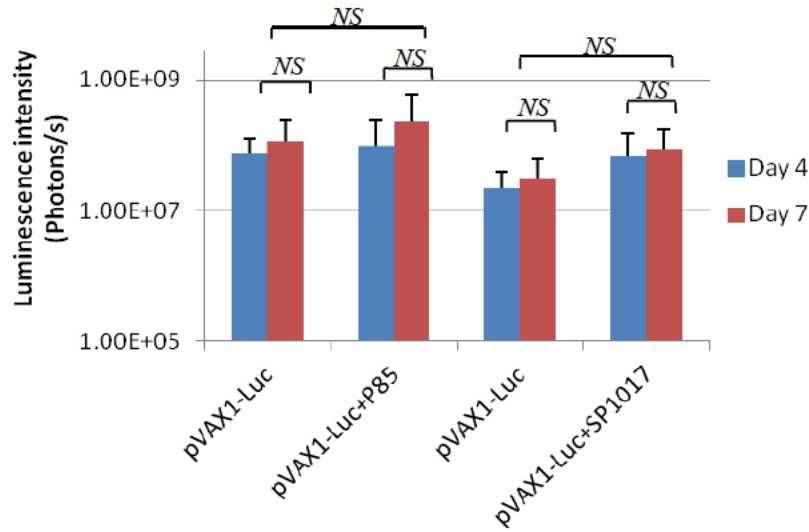
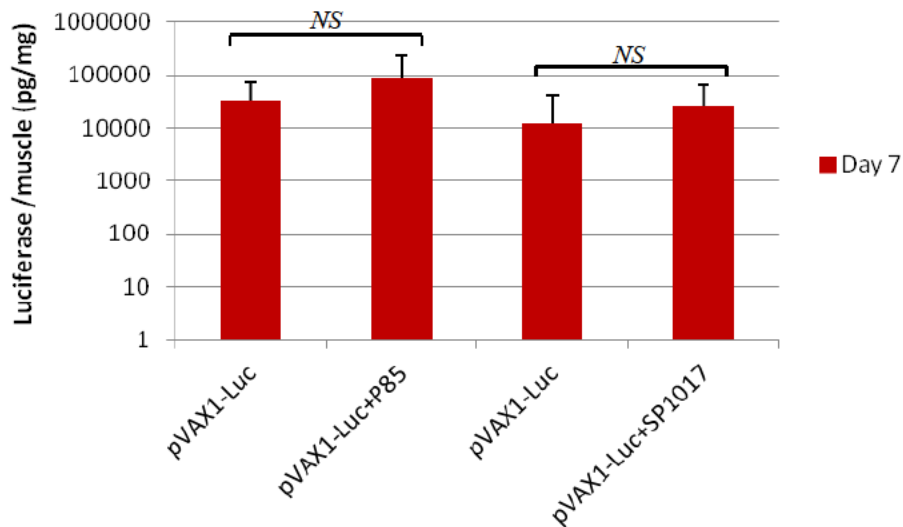
B



**Figure 16.** *In vivo* pVAX1-Luc plasmid expression in bilateral tibialis anterior muscles in BALB/c mice (5 mice/group) after a single administration of 10  $\mu$ g pDNA formulated with (R, Right tibialis anterior muscles) /without (L, Left tibialis anterior muscles) 0.3% (w/v) P85 in 50  $\mu$ l PBS. **A.** *In vivo* images were taken on day 4 (D4) and day 7 (D7) after i.m. injection using the Xenogen In Vivo Imaging System-IVIS 200. **B.** Quantitative data of *in vivo* imaging study for each mouse.



**Figure 17.** *In vivo* pVAX1-Luc plasmid expression in bilateral tibialis anterior muscles in BALB/c mice (5 mice/group) after a single administration of 10  $\mu$ g pDNA formulated with (R, Right tibialis anterior muscles) /without (L, Left tibialis anterior muscles) 0.01% (w/v) SP1017 in 50  $\mu$ l PBS. **A.** *In vivo* imaging was performed on day 4 (D4) and day 7 (D7) after i.m. injection using the Xenogen In Vivo Imaging System-IVIS 200. **B.** Quantitative data of *in vivo* imaging study for each mouse.

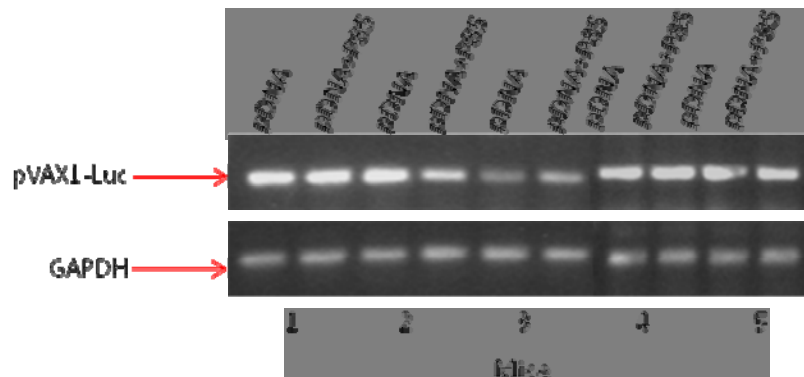
**A****B**

**Figure 18.** Average luminescence intensity of mice administrated with 10  $\mu$ g of pVAX1-Luc pDNA formulated with/without 0.3% w/v P85 or 0.01% SP1017 in tibialis anterior muscles in BALB/c mice (5 mice/ group). Data are mean  $\pm$ SD (n=5). *P* values were obtained by the means of Student's *t* test. Each *P* value corresponds to the comparison of naked DNA versus DNA formulated with P85 or 0.01% SP1017 and Day 4 versus Day 7: *NS* is not significant at 0.05 levels. (A) *In vivo* imaging was done on day 4 and day 7 after i.m. injection using the Xenogen In Vivo Imaging System-IVIS 200. (B) Tibialis anterior muscles were collected on day 7 after single i.m. injections and *in vivo* imaging and used for analysis of the luciferase activity.

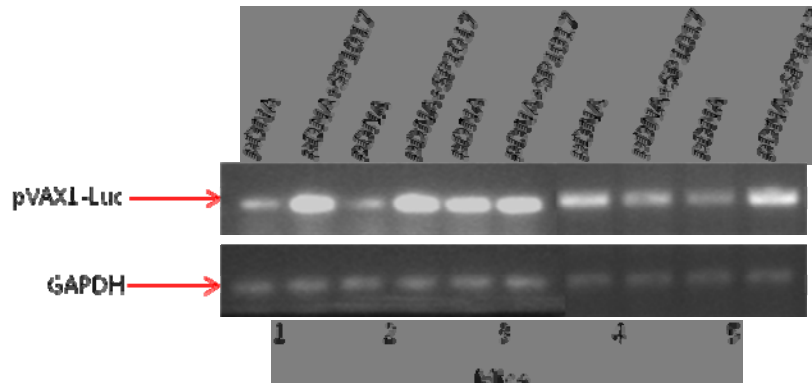
To determine the transgene expression of Luciferase and H5HA at mRNA level, 10  $\mu$ g of pVAX1-H5HA and pVAX1-Luc (control plasmid) pDNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered in the tibialis anterior muscles of BALB/c mice (5 mice/ group). On day 7, mice were sacrificed and tibialis anterior muscles were collected to

perform the RT-PCR. mRNA levels were normalized to that of GAPDH. mRNA levels of Luciferase were determined in all 5 mice in both groups treated with/without P85 or SP1017 (**Figure 19A and B**). However, the group treated with/without 0.01% w/v SP1017 expressed higher level of mRNA of luciferase compared to that of P85 (**Figure 19**). Expression levels of luciferase at mRNA levels also varied in all 5 mice in both groups. The similar results also observed in case of H5HA transcription. H5HA expression was observed in all 5 mice in both groups treated with/without 0.3% w/v P85 or 0.01% w/v SP1017 (**Figure 20**). The variations in transcription of H5HA were also observed in both groups (**Figure 20A and B**). However, expressions of H5HA at mRNA levels were higher in the group treated with/without SP1017 compared to that of P85. Co-administration of both Pluronics with pDNA increased the transcription of H5HA at mRNA levels compared to the naked pDNA (**Figure 20**).

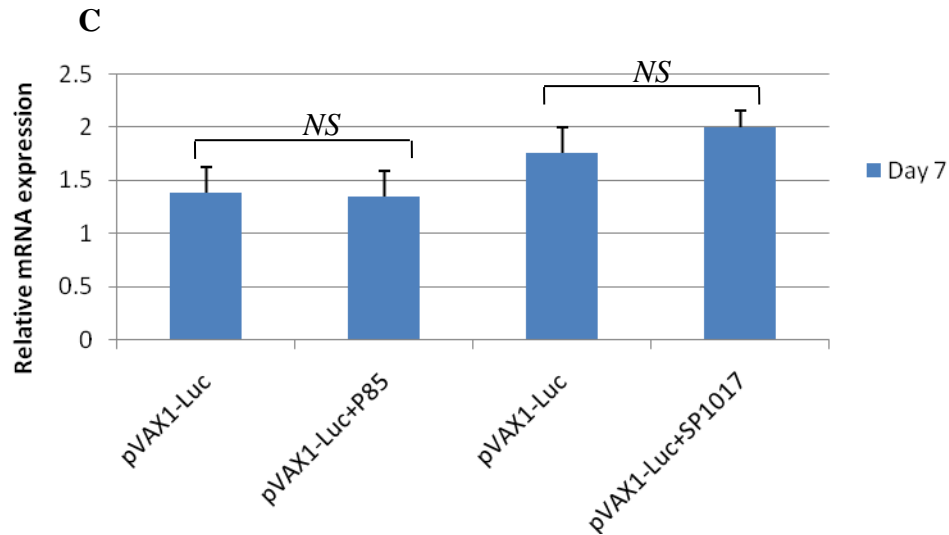
**A**



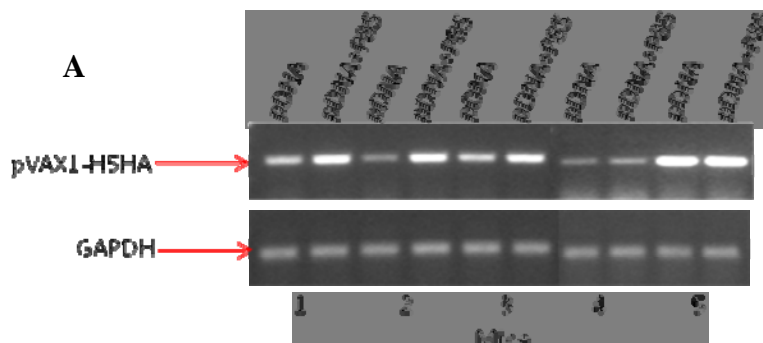
**B**

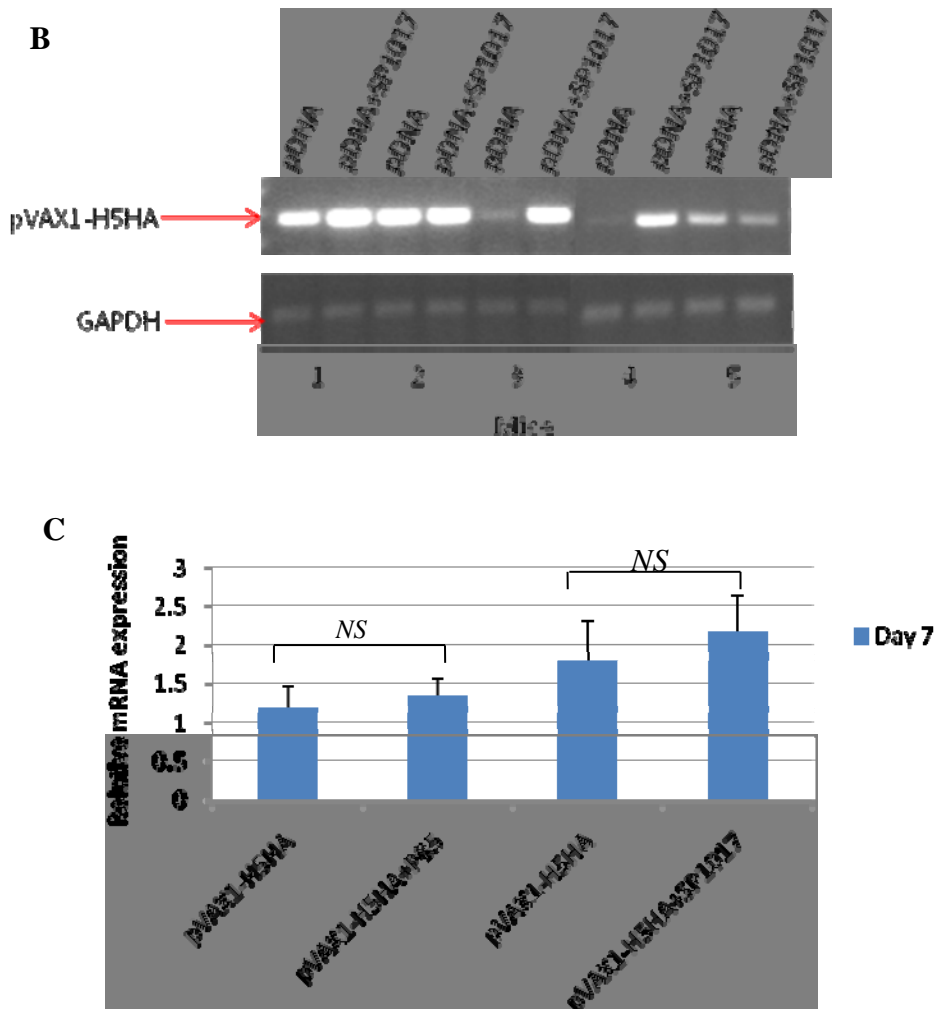






**Figure 19.** Effects of Pluronics on mRNA levels of Luciferase gene. 10  $\mu$ g of pVAX1-Luc pDNA formulated with/without 0.3 % w/v P85 or 0.01% w/v SP1017 were administered in tibialis anterior muscles of BALB/c mice (5 mice/group). Tissue samples were collected on day 7 and used for the RT-PCR. RT-PCR products of Luciferase (230 bp) (n=5) from P85 treated group (**A**) and from SP1017 group (**B**) were run by electrophoresis on a 2% agarose gel containing ethidium bromide. The images were acquired by using Gel Doc (Bio-Rad) and analyzed by ImageJ software (NIH). (**C**) mRNA levels for Luciferase gene expression normalized with respect to GAPDH and expressed as arbitrary units. Data are reported as means  $\pm$ SD (n=5). *P* values were obtained by the means of Student's *t* test. Each *P* value corresponds to the comparison of naked DNA versus DNA formulated with P85 or SP1017: *NS* is not significant at 0.05 levels.





**Figure 20.** Effects of Pluronics on mRNA levels of H5HA gene expression. 10  $\mu$ g of pVAX1-H5HA pDNA formulated with/without 0.3 % w/v P85 or 0.01% w/v SP1017 were administered in tibialis anterior muscles of BALB/c mice (5 mice/group). Tissue samples were collected on day 7 and used for the RT-PCR. The RT-PCR products of H5HA expression (230 bp, n=5) from P85-treated (**A**) and SP1017-treated (**B**) groups were run by electrophoresis on a 2% agarose gel containing ethidium bromide. The images were acquired by using Gel Doc (Bio-Rad) and analyzed by ImageJ software (NIH). (**C**) mRNA levels for H5HA expression normalized with respect to GAPDH and expressed as arbitrary units. Data are reported as means  $\pm$ SD (n=5). *P* values were obtained by the means of Student's *t* test. Each *P* value corresponds to the comparison of naked DNA versus DNA formulated with P85 or SP1017: NS is not significant at 0.05 levels.

The effects of 0.3% w/v P85 and 0.01% w/v SP1017 formulated pDNA of Luciferase and H5HA on transgene expression after i.m. administration in the tibialis anterior muscles in mice are summarized on **Table 5**.

**Table 5.** Effects of 0.3% w/v P85 and 0.01% w/v SP1017 formulated pDNA of Luciferase and H5HA on transgene expression after i.m. administration in tibialis anterior muscles in BALB/c mice on day 7.

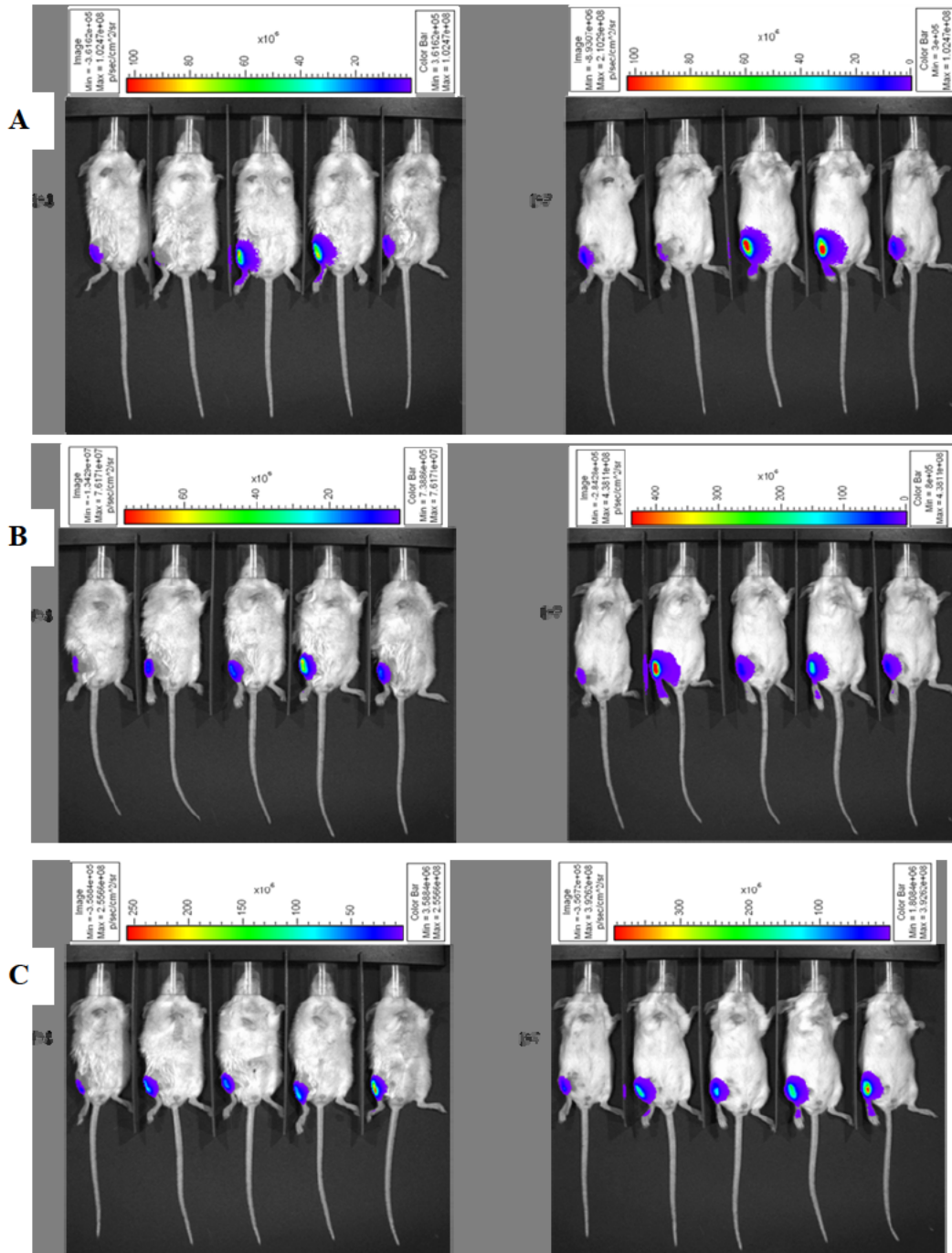
	<i>In vivo</i> luciferase expression Luminescence intensity (Photons/s)		<i>Ex vivo</i> luciferase expression Luciferase/muscle (ng/mg)		Luciferase expression at mRNA level Luciferase/GAPDH (Arbitrary units)		H5HA expression at mRNA level H5HA/GAPDH (Arbitrary units)	
	pDNA	pDNA +Pluronic	pDNA	pDNA +Pluronic	pDNA	pDNA +Pluronic	pDNA	pDNA +Pluronic
<b>P85 group</b> (Fold Increase)	1.15E+08 (1.0)	2.26E+08 (1.97)	33.88 (1.0)	86.51 (2.56)	1.38 (1.0)	1.34 (0.97)	1.19 (1.0)	1.36 (1.15)
Significance		NS		NS		NS		NS
<b>SP1017 group</b> (Fold Increase)	2.96E+07 (1.0)	8.37E+07 (2.83)	12.43 (1.0)	25.97 (2.09)	1.76 (1.0)	2.0 (1.14)	1.81 (1.0)	2.17 (1.2)
Significance		NS		NS		NS		NS

NS: data are not statistically significant

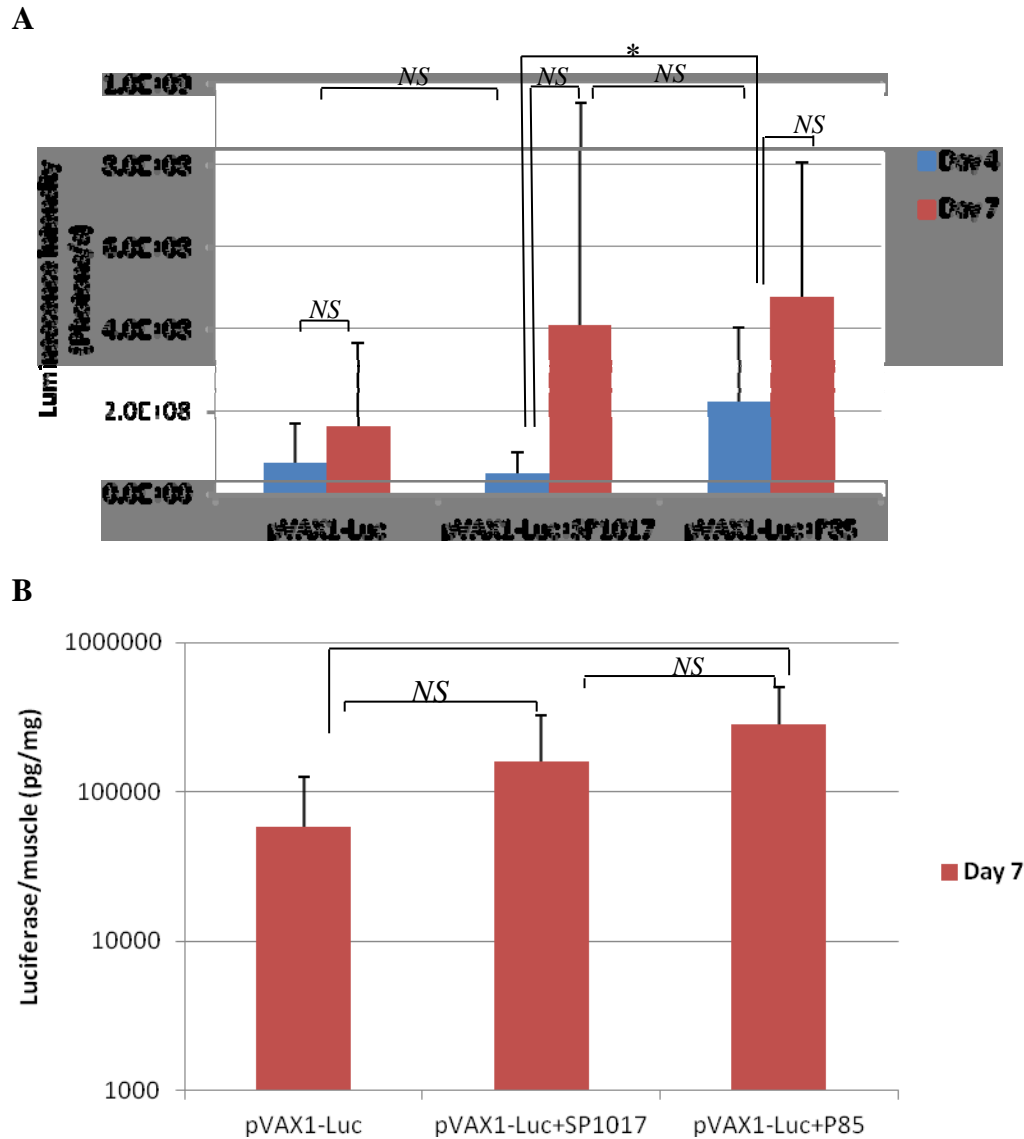
To avoid the combined effects of plasmid DNA and Plurionics on transgene expression in mice, plasmid DNA formulations with/without Plurionics block copolymers were administered in only right tibialis anterior muscles of BALB/c mice.

In a first set of experiments, 10 µg of pVAX1-Luc (control plasmid) plasmid DNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered i.m. in tibialis anterior muscles in BALB/c mice (5 mice/group). The animals were imaged on day 4 and day 7 after i.m. administration (**Figure 21** and **22**). Luciferase expression in skeletal muscles was detected on day 4 in all 5 mice after single injection of pVAX1-Luc pDNA. The level of expression was further elevated on a day 7 after injection (**Figure 15A** and **16A**). Similar results were also obtained with pDNA formulated with 0.3% w/v P85 or 0.01% w/v SP1017 (**Figure 21B, C** and **22A**). Tibialis anterior muscles were collected on day 7 and luminescence assay was performed on muscle tissue to determine the luciferase activity (**Figure 22B**).

As it seen from the presented data, formulation of pVAX1-Luc pDNA with Pluronic copolymers (either Plurionics P85 or SP1017) enhances transgene expression in mice (**Figure 21** and **22**). To compare the effects of Plurionics on transgene expression of pVAX1-Luc, the average intensity of luminescence were plotted in **Figure 22**. Transfection with pDNA in combination with P85 led to higher expression of encoded gene compared to pDNA formulated with SP1017 *in vivo* (**Figure 22**).



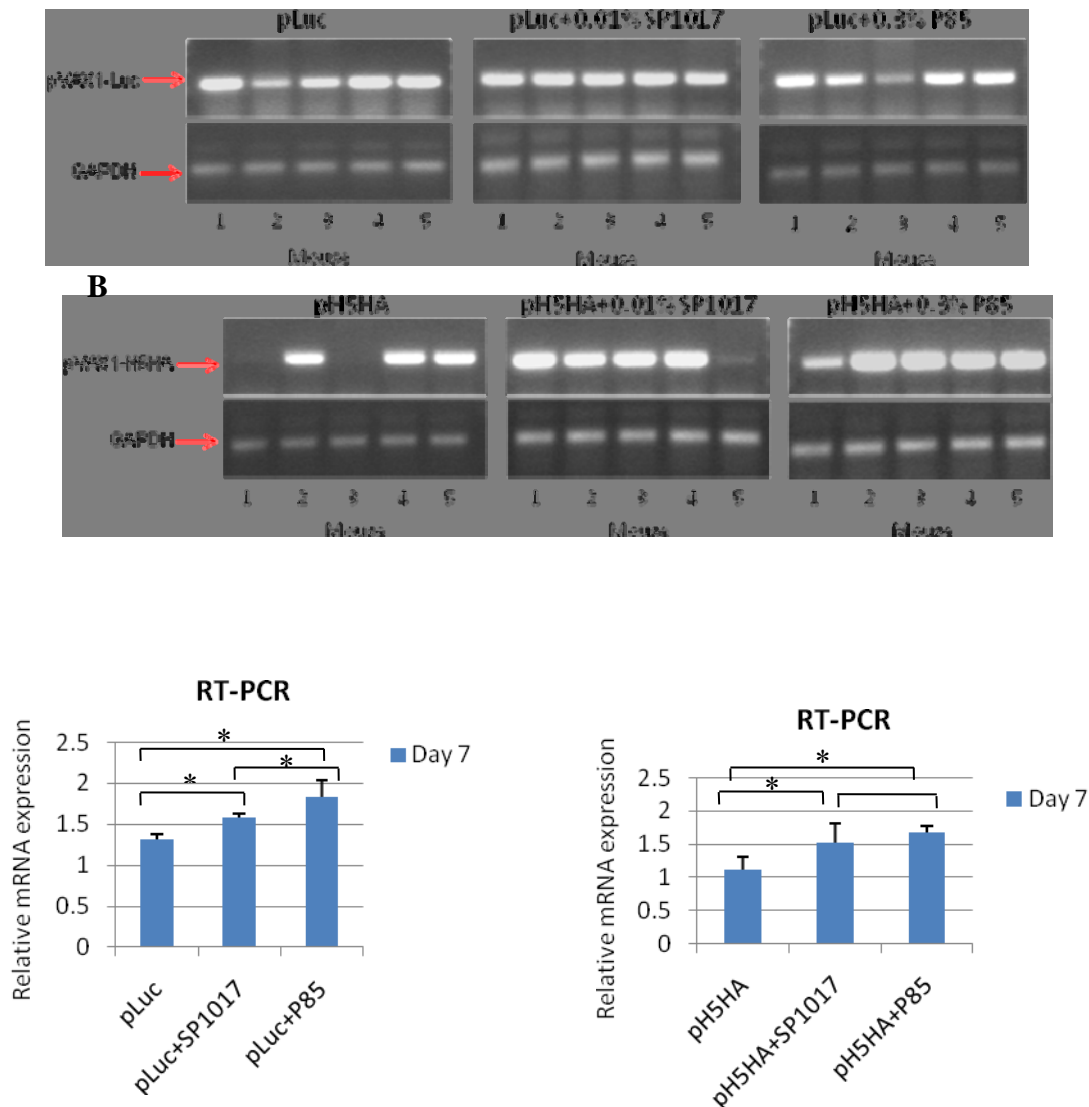
**Figure 21.** *In vivo* pVAX1-Luc plasmid expression in tibialis anterior muscles in BALB/c mice (5 mice/group) after a single administration of 10  $\mu$ g pVAX1-Luc pDNA formulated with /without 0.01% (w/v) SP1017 or 0.3% (w/v) P85 in 50  $\mu$ l PBS **A.** pDNA only, **B.** pDNA with 0.01% SP1017; and **C.** pDNA with 0.3% (w/v) P85. *In vivo* imaging was performed on day 4 (D4) and day 7 (D7) after i.m. injection using the Xenogen In Vivo Imaging System-IVIS 200.



**Figure 22.** (A) Average luminescence intensity in the muscle of mice injected with pVAX1-Luc pDNA formulated with/without 0.3% w/v P85 or 0.01% SP1017. *In vivo* imaging was performed on day 4 and day 7. (B) Average luciferase activity in muscle tissue on day 7 after single i.m. injections. Data are presented as mean  $\pm$  SD (n=5). *P* values were obtained by the means of Student's *t* test. \**P*<0.05, NS : the differences are not statistically significant.

To determine the transgene expression of luciferase and H5HA at mRNA level, 10  $\mu$ g of pVAX1-H5HA and pVAX1-Luc (control plasmid) pDNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were i.m. injected to BALB/c mice (5 mice/ group). On day 7, mice were sacrificed and tibialis anterior muscles were collected to perform the RT-PCR. mRNA levels were normalized to that of GAPDH. Transgene mRNA levels were detected in all mice

injected with pVAX1-Luc pDNA or its formulations with Pluronic copolymers. However, H5HA mRNA levels varied in the groups treated with pVAX1-H5HA plasmid (**Figure 23A and B**). pVAX1-Luc pDNA formulated with 0.3% w/v P85 or 0.01% w/v SP1017 produced significantly higher luciferase expression at mRNA level in all 5 mice on day 7 compared to that of naked pDNA (**Figure 23A and C**). The similar results were also observed in case of H5HA transcription (**Figure 23B and D**). Expressions of H5HA at mRNA levels were higher in the group treated with P85 compared to that of SP1017. These results suggest that co-administration of both Pluronics with pDNA increased the transcription of Luciferase and H5HA at mRNA levels compared to that of naked pDNA (**Figure 23**).



**Figure 23.** Effects of Pluronics on mRNA levels of Luciferase and H5HA gene expression. RT-PCR products of (A) Luciferase (230 bp) and (B) H5HA (230 bp) gene expression were run on 2% agarose gel containing ethidium bromide. The images were acquired by using Gel Doc (Bio-Rad) and analyzed by

ImageJ software (NIH). (C) and (D) mRNA levels for luciferase and H5HA gene normalized with respect to GAPDH and expressed in arbitrary units. Data are presented as mean  $\pm$ SD (n=5). *P* values were obtained by the means of Student's *t* test. \**P*<0.05, *NS* : the differences are not statistically significant.

The effects of 0.3% w/v P85 and 0.01% w/v SP1017 on transgene expression after i.m. administration in mice are summarized in **Table 6**.

**Table 6.** Effects of 0.3% w/v P85 and 0.01% w/v SP1017 formulated pDNA of Luciferase and H5HA on transgene expression after i.m. administration in tibialis anterior muscles in BALB/c mice on day 7.

	<i>In vivo</i> luciferase expression Luminescence intensity (Photons/s)	<i>Ex vivo</i> luciferase expression Luciferase/muscle (ng/mg)	Luciferase expression at mRNA level Luciferase/GAPDH (Arbitrary units)	H5HA expression at mRNA level H5HA/GAPDH (Arbitrary units)
<b>pDNA only group</b>	1.63E+08	57.77	1.32	1.12
(Fold increase)	(1.0)	(1.0)	(1.0)	(1.0)
<b>pDNA/SP1017 group</b>	4.11E+08	158.90	1.58	1.52
(Fold increase)	(2.52)	(2.75)	(1.2)	(1.36)
Significance	<i>NS</i>	<i>NS</i>	* <i>P</i> <0.05	* <i>P</i> <0.05
<b>pDNA/P85 group</b>	4.79E+08	280.18	1.83	1.68
(Fold increase)	(2.93)	(4.85)	(1.4)	(1.5)
Significance	<i>NS</i>	<i>NS</i>	* <i>P</i> <0.05	* <i>P</i> <0.05

*NS*: data are not statistically significant

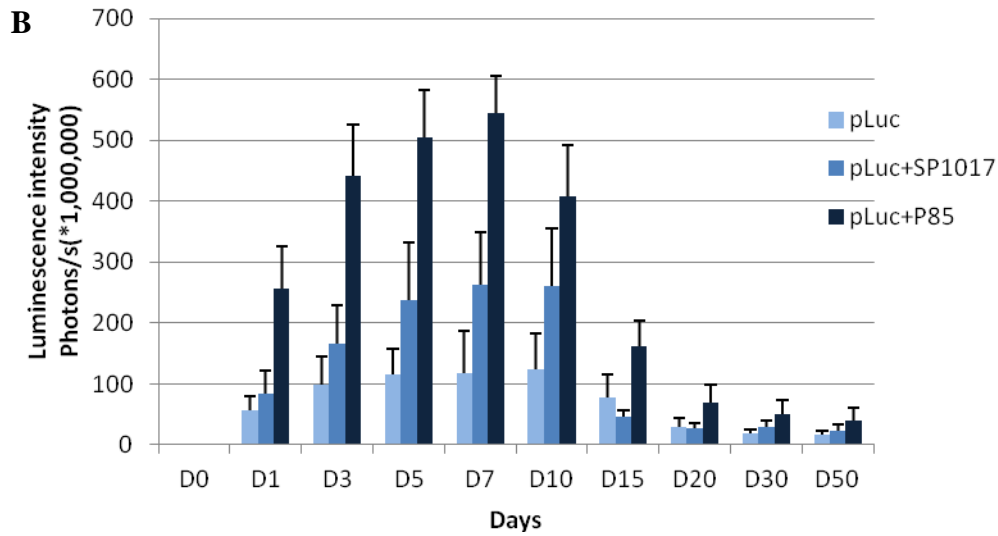
Next, in order to compare the effect of Pluronic SP1017 with P85 on the level and prolongation of transgene expression of H5HA and Luciferase gene in tibialis anterior muscles in BALB/c mice, 10  $\mu$ g of plasmid DNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered. The following experiments were performed to determine the Luciferase and H5HA expression *in vivo*.

In a first set of experiments, 10  $\mu$ g of pVAX1-Luc (control plasmid) plasmid DNA (pDNA) formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered i.m. in tibialis anterior muscles in BALB/c mice (10 mice/group). The animals were subjected to non-invasive bioluminescence detection using the Xenogen In Vivo Imaging System-IVIS 200 on day 0, 1, 3, 4, 5, 7, 10, 15, 20, 30, and 50 after i.m. administration (**Figure 24A**). Transgene expression was greatly prolonged and lasted for at least 50 days for Luciferase gene (**Figure 24A**). 10  $\mu$ g of naked pVAX1-Luc pDNA produced luciferase expression in skeletal muscles with higher intensity on day 10 however as it seen from the presented data, formulation of pVAX1-Luc pDNA with Pluronic copolymers (either Pluronic P85 or SP1017) enhances transgene expression in mice on day 7 (**Figure 24A and B**). Tibialis anterior muscles were also collected on day 4 and luminescence assay was performed on muscle tissue to determine the luciferase

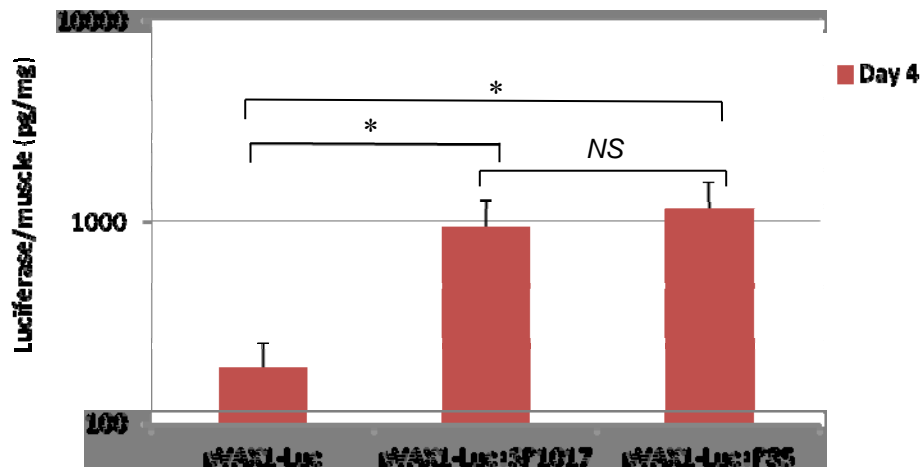








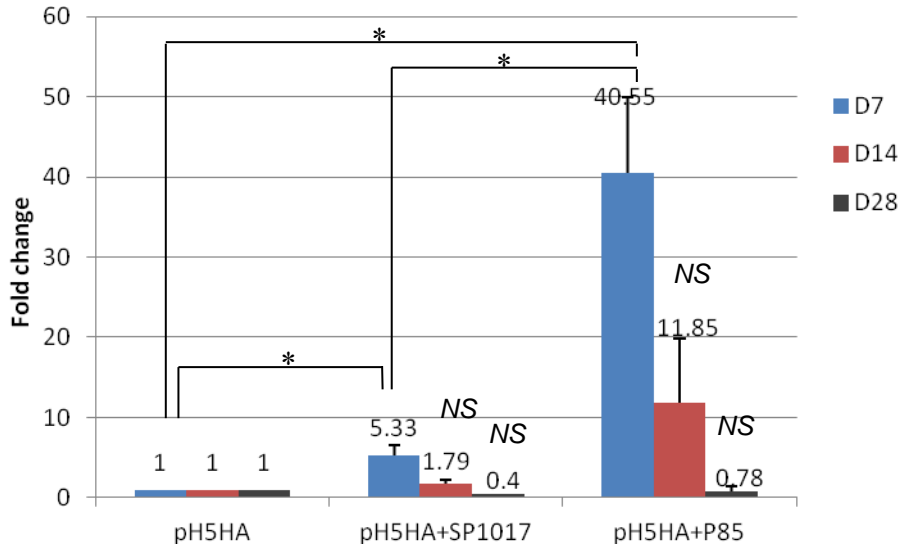
**Figure 24.** A. *In vivo* pVAX1-Luc plasmid expression in tibialis anterior muscles in BALB/c mice (10 mice/group) after a single administration of 10  $\mu$ g pVAX1-Luc pDNA formulated with /without 0.01% (w/v) SP1017 or 0.3% (w/v) P85 in 50  $\mu$ l. *In vivo* imaging was done on until day 50 (D50) after i.m. injection using the Xenogen In Vivo Imaging System-IVIS 200. B. Average luminescence intensity of mice. Data are mean  $\pm$ SEM (n=10).



**Figure 25.** Average luminescence intensity of mice administrated with 10  $\mu$ g of pVAX1-Luc pDNA formulated with/without 0.3% w/v P85 or 0.01% SP1017 in tibialis anterior muscles in BALB/c mice (10 mice/ group). Data are mean  $\pm$ SEM (n=10). *P* values were obtained by the means of Student's *t* test. Each *P* value corresponds to the comparison of naked DNA versus DNA formulated with P85 or 0.01% SP1017 on Day 4. \**P*<0.05, NS is not significant at 0.05 levels. Tibialis anterior muscles were collected on day 4 and used for analysis of the *ex-vivo* luciferase activity.

**Expression of H5HA gene at mRNA levels:** To determine the transgene expression and prolongation of H5HA at mRNA level, 10 µg of pVAX1-H5HA pDNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered in tibialis anterior muscles of BALB/c mice (5 mice/ group). To determine the level and prolongation of H5HA expression, tibialis anterior muscles were collected to perform the Real-time RT-PCR on day 7, 14 and 28 after administration of the formulations. RNA were extracted from the harvested muscles using RNeasy Fibrous Tissue Mini kit according to the manufacturer's protocol (QIAGEN, Valencia, CA). RNA samples were quantified by NanoDrop 2000 spectrophotometer (Thermo Fisher, Waltham, MA). 3.5 µg of total DNase-treated RNA from each sample were used for cDNA synthesis. Reverse transcription to prepare cDNA was performed with Oligo(dT) primers (Invitrogen, Carlsbad, CA) and Superscript II (Invitrogen, Carlsbad, CA). RNaseOUT (Invitrogen, Carlsbad, CA) was used to inhibit ribonuclease activity during reaction sequences. Efficient cDNA synthesis was accomplished by incubation of total RNA for 90 min at 42°C followed by 5 min denaturation at 94°C. The cDNAs were further used for the Real-time RT-PCR. HA specific influenza virus H5 and GAPDH primers and probes were designed for the H5 and GAPDH TaqMan Real-time assay by using Primer Express software (Applied Biosystems, Branchburg, NJ). Primers and probes were H5 Forward primer 5'GAG ATT GTA GCG TAG CTG GAT GG 3', H5 Reverse primer 5'CAT TCC GGC ACA TTG ATG AA 3', TaqMAN H5 probe 5' TCC TCG GAA ACC CAA TGT GTG ACG A 3', GAPDH Forward primer 5' ACT GGC ATG GCC TTC CG 3', GAPDH Reverse primer 5' CAG GCG GCA CGT CAG ATC 3' and TaqMan GAPDH probe 5' TTC CTA CCC CCA ATG TGT CCG TCG T 3' (Sigma, St. Louis, MO). Two microliters of cDNA samples were used per 20 µL reaction volume containing 5 µL 2X Taqman Universal PCR Master Mix (Applied Biosystems, Branchburg, NJ), and 250 nM of each primer and probe. All samples and controls were tested in triplicate. The PCR thermal profile consisted of 50°C for 2 minutes, 95°C for 10 minutes, and then 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Amplification, detection, and data analysis were performed on an ABI Prism 7000 Real-time thermal cycler (Applied Biosystems, Branchburg, NJ) with "FAM- Tamra quencher" selected as the detector. Relative expression of H5HA was normalized to expression of GAPDH. Relative quantification of gene expression was calculated based on the comparative CT (threshold cycle value) method ( $\Delta CT = CT \text{ gene of interest} - CT \text{ housekeeping gene}$ ). Comparison of gene expression in different samples was performed based on the differences in  $\Delta CT$  of individual samples ( $\Delta\Delta CT$ ) and the fold difference was calculated as  $2^{\Delta\Delta CT}$ . All data are presented as means  $\pm$  standard error (SEM) and analyzed with Student's *t*-test (*n*=3). Differences were accepted as statistically significant at *p* < 0.05. H5HA gene transcription were determined in all 5 mice in all 3 groups through day 28 though transcriptions of H5HA varied in those groups. In this study, we incorporated 3 most positive reactions in Real-time RT-PCR assay to calculate the data from 3 out of 5 mice per group. pVAX1-H5HA pDNA formulated with 0.3% w/v P85 or 0.01% w/v SP1017 produced significantly higher H5HA gene expression at mRNA levels on day 7 compared to naked pDNA (**Figure 26**). Compared to naked pDNA, 0.01% SP1017 and 0.3% P85 increased the transcription of H5HA by 5.3 and 40.6 fold on day 7 in tibialis anterior muscles. However, H5HA gene transcription levels were decreased by days. On day 15, SP1017 and P85 increased the mRNA levels by 1.8 and 11.9 fold respectively. Whereas, fold induction by SP1017 and P85 were by 0.4 and 0.8 fold respectively compared to naked pDNA on day 28. Expression of H5HA at mRNA levels was always higher in the group treated with P85 compared to SP1017. These results suggest that administration of pVAX1-H5HA pDNA prolonged the transcription until day

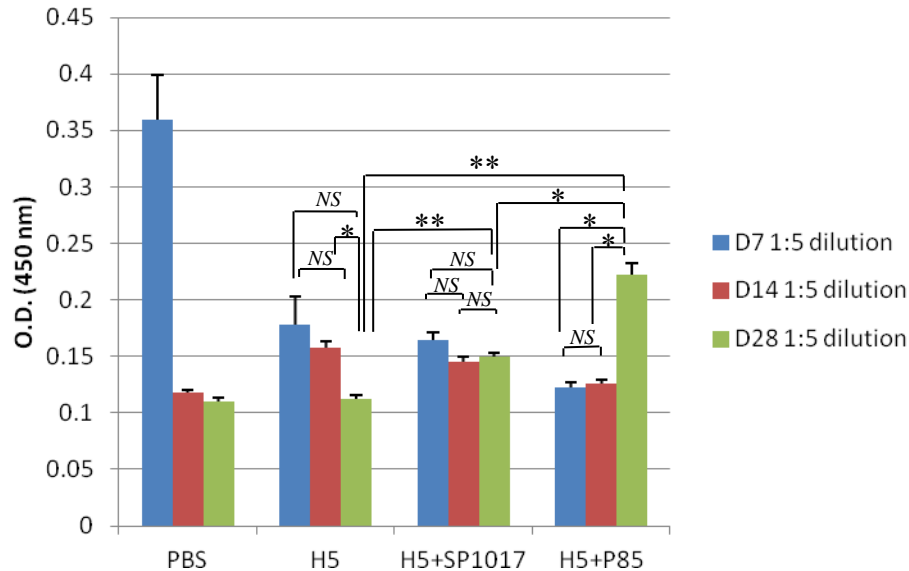
28 and co-administration of both Pluronics with pDNA increased the transcription levels compared to naked pDNA (**Figure 26**).



**Figure 26.** mRNA levels of H5HA gene determined by Real-time RT-PCR. BALB/c mice administrated with 10  $\mu$ g of pVAX1-H5HA pDNA formulated with/without 0.3% w/v P85 or 0.01% SP1017 in tibialis anterior muscles (5 mice/ group). Tibialis anterior muscles were collected on day 7 (D7), day 14 (D14) and day 28 (D28) after administration of the formulations and performed Real-time RT-PCR. Induction ratios of H5HA gene (fold change) by Pluronics were calculated using expression level, normalized to the level of the naked pDNA group. Data are mean  $\pm$  SEM (n=3). *P* values were obtained by the means of Student's *t* test. Each *P* value corresponds to the comparison of naked DNA versus DNA formulated with P85 or 0.01% SP1017. \**P*<0.05, NS is not significant at 0.05 levels.

We were focused on evaluating whether a single immunization with Pluronic/pVAX1-H5HA DNA vaccine can produce an immune response.

In order to determine the immune response, 10  $\mu$ g of pVAX1-H5HA pDNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered i.m. in tibialis anterior muscles in BALB/c mice (5 mice/group). H5HA specific antibodies in blood sera were detected by enzyme-linked immunosorbent assay (ELISA). Blood sera were collected on day 7, 14 and 28 after i.m. injection. H5HA protein was purified from HEK-293T cells transfected with pH5HA using MagneHis Protein Purification System according to manufacturer's protocol (Promega, Madison, WI). 96-well microtiter plates (Nunc Life Technologies, Rochester, NY) were coated with 2  $\mu$ g/mL purified H5HA in PBS overnight at 4°C. Plates were blocked for 1 h at room temperature with 1 % BSA/ PBS. 100  $\mu$ L of serum dilutions were added to the plates and incubated for 2 h at room temperature. Plates were washed five times, and HRP conjugated mouse IgG detection antibody (Bethyl Laboratories, Inc. Montgomery, TX) diluted 1:50,000 was added to wells followed by 1 h incubation at room temperature. TMB peroxidase substrate (Bethyl Laboratories, Inc. Montgomery, TX) then added to the wells and allowed to develop for 15 min at room temperature. Optical density measurements were done at 450nm.



**Figure 27.** Total serum IgG antibody responses to H5HA.10  $\mu$ g of pVAX1-H5HA pDNA formulated with/without 0.01% (w/v) SP1017 or 0.3% (w/v) P85 in 50  $\mu$ L of PBS were administered in tibialis anterior muscles in BALB/c mice (5 mice/group). Sera from individual mice were taken 7, 14 and 28 days after single administration of vaccine formulations and were tested for binding to H5 hemagglutinin by ELISA. All data are presented as means  $\pm$  standard error (SEM) and analyzed with Student's *t*-test ( $n=5$ ). Each *P* value corresponds to the comparison of naked DNA versus DNA formulated with P85 or 0.01% SP1017 \*\* $P < 0.01$  significant at 0.01 levels. \* $P < 0.05$  and NS is not significant at 0.05 levels.

In order to determine H5HA specific antibody levels in sera after single i.m. administration of pH5HA in combination with Pluronics in the tibialis anterior muscles of BALB/c mice (5mice/group) were determined by enzyme-linked immunosorbent assay (ELISA). The single immunization of mice with pH5HA formulations induced low but detectable levels of total IgG antibodies as measured by ELISA plates coated with H5HA protein (**Figure 27**). The levels of virus-specific antibodies were significantly increased after 28 days in the groups immunized with Pluronics SP1017 and P85 compared to naked DNA (**Figure 27**). The non-specific anti-H5HA IgG were also detected in PBS group probably due to the presence of highly conserved amino acid sequences of pocket on the stem of HA (**Figure 27**).

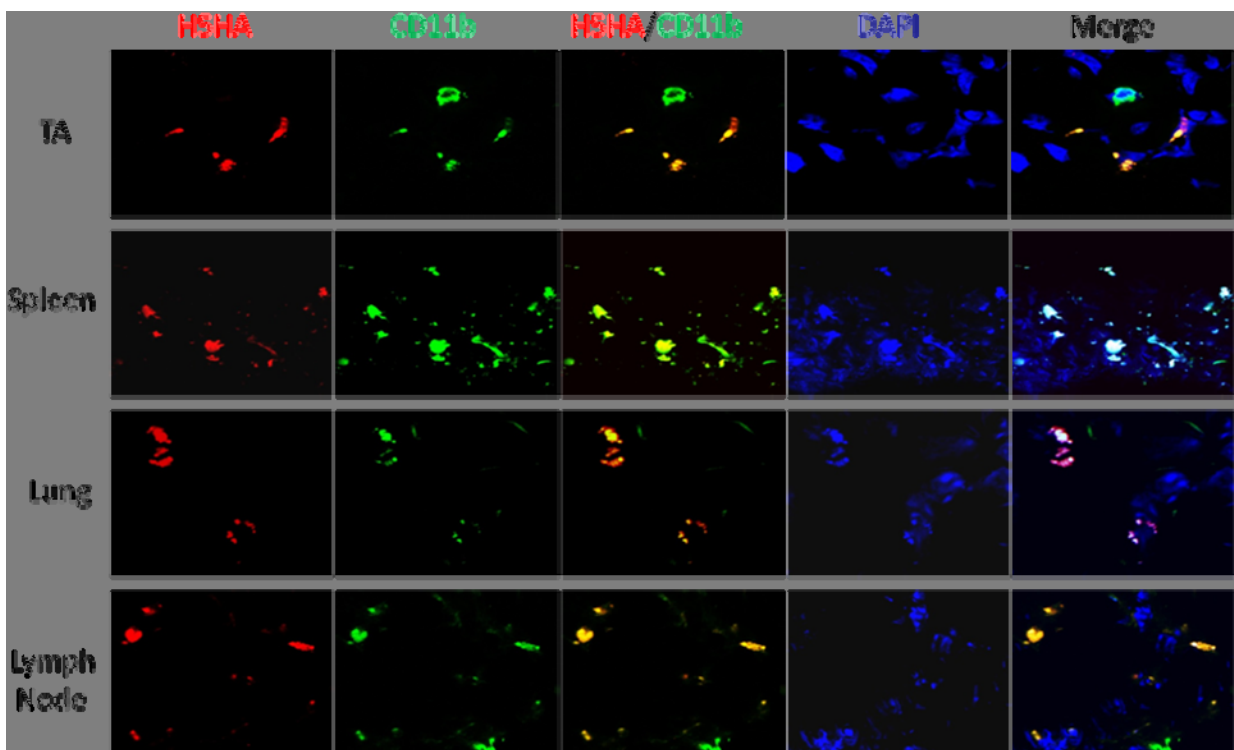
### **Year 3**

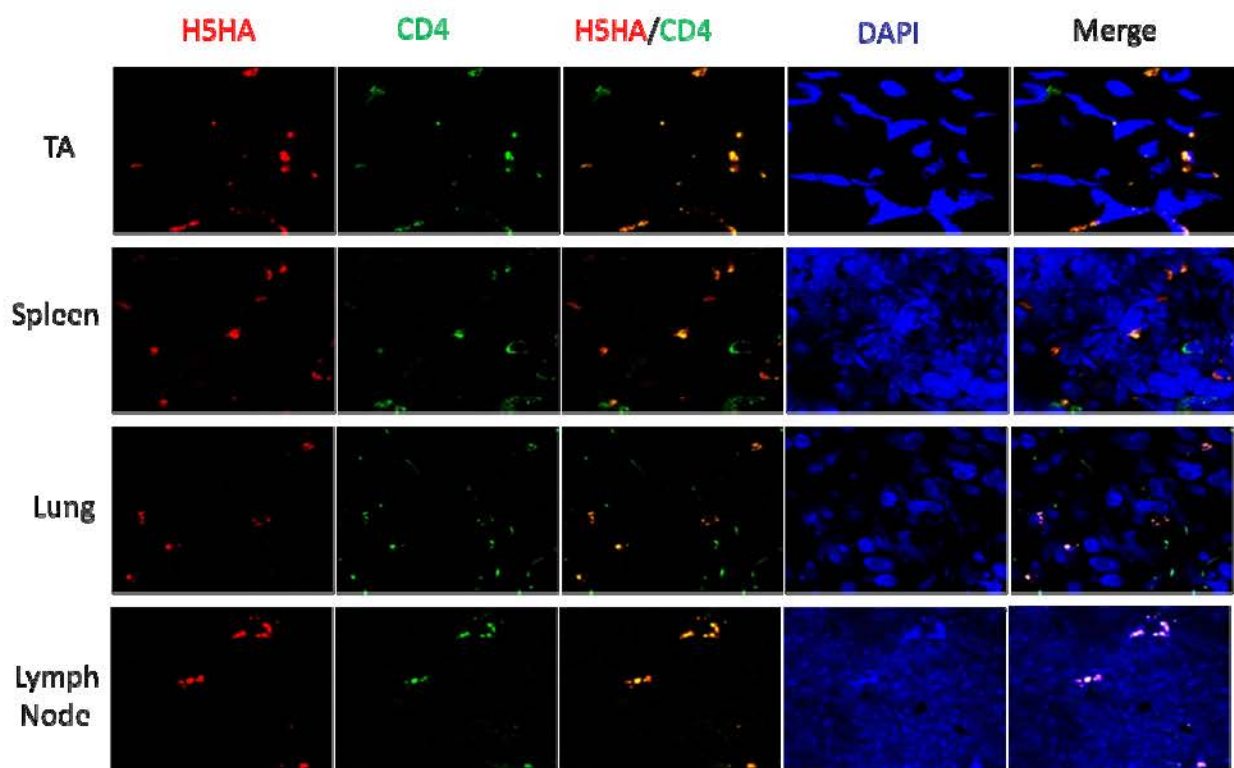
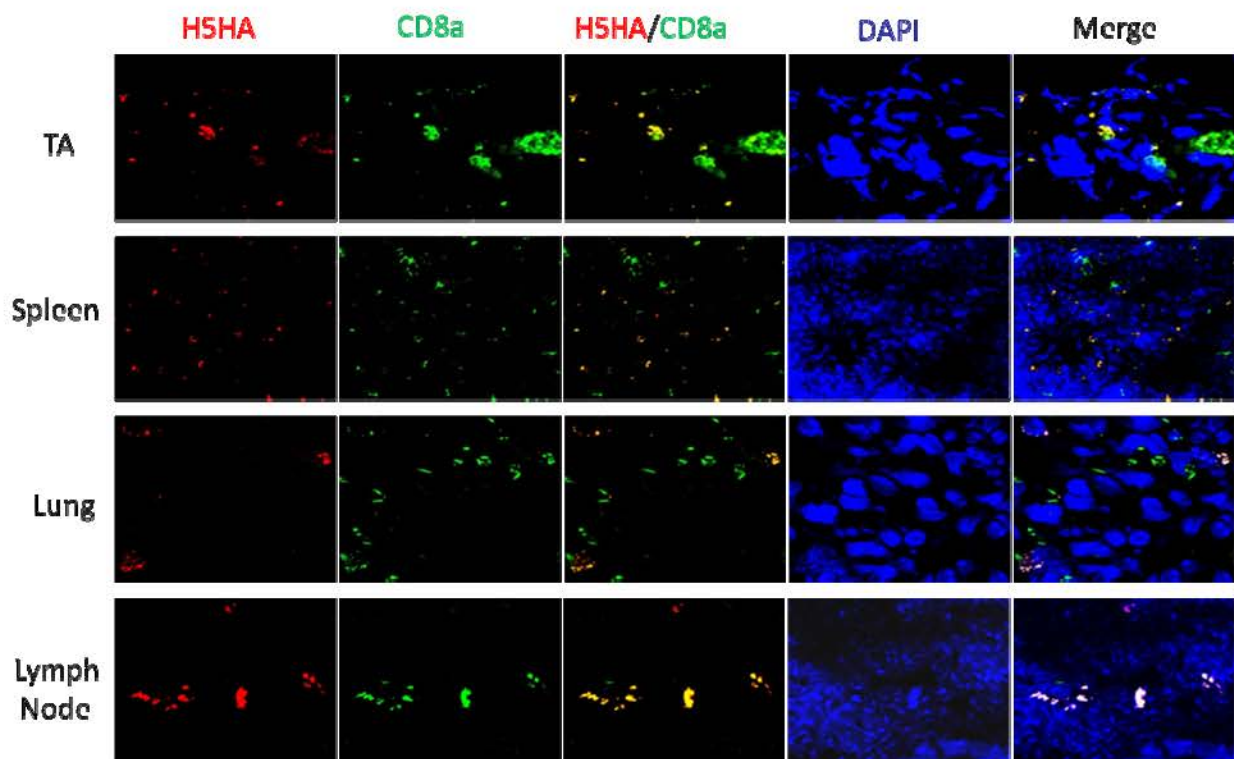
In support of the design of DNA plasmid encoding influenza protein H5N1 HA, the DNA sequence coding therapeutic gene H5HA has been successfully constructed into the pVAX1 backbone. In order to develop effective vaccine formulations, the goal of this experiment is to determine what cell populations accumulate the DNA and express the transgene after administration of the pVAX1-H5HA plasmid DNA in the skeletal muscle in a mouse.

10  $\mu$ g of pVAX1-H5HA plasmid DNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered i.m. in tibialis anterior muscles in BALB/c mice (5 mice/group). The animals were sacrificed on day 7 after i.m. injection. Tibialis anterior muscle, lymph node, spleen and lung were harvested, and embedded in Tissue-Tek OCT, rapidly cooled and stored at  $-80^{\circ}\text{C}$  for performing immunohistochemistry. Double staining immunofluorescence was performed in frozen tissue sections cut on plain glass slides. The glass slides were sequentially

treating with a) monoclonal mouse anti-His-tag antibody (Cellsignaling, Danvers, MA) to detect His-tagged H5HA antigen, cell-specific Abs for macrophages (CD11b) (eBioscience, San Diego, CA), cytotoxic T cells (CD8a) (BioLegend, San Diego, CA) and T-helper cells (CD4) (Abcam, Cambridge, MA). Specifically, 12  $\mu$ m frozen sections of tissues (four slices per tissue specimen) were fixed in ice cold methanol and then blocked for 10 mins in 5% BSA in Tris-buffered saline at room temperature, rinsed two times and incubated overnight at 4°C with anti-His-tag and cell marker primary antibodies. After that the sections were rinsed three times with PBS and incubated 1 h at room temperature with secondary anti-species (rat/mouse) antibodies conjugated to a Alexa Fluor 594 (red)/Alexa Fluor 488 (green) (Invitrogen, Carlsbad, CA) were used. Two negative control specimens (PBS injected mice) were also used in this protocol. Finally, the samples were counterstained with DAPI and analyzed by Zeiss 710 Confocal Laser Scanning Microscope equipped with an Argon-Krypton Laser 595 nm (H5HA expression), 488 nm (cell marker) and 647 nm (nucleus) to visualize the co-localization (yellow) of HA (red) and cell-specific marker (green). The images were digitally superimposed to determine which cells express the transgene.

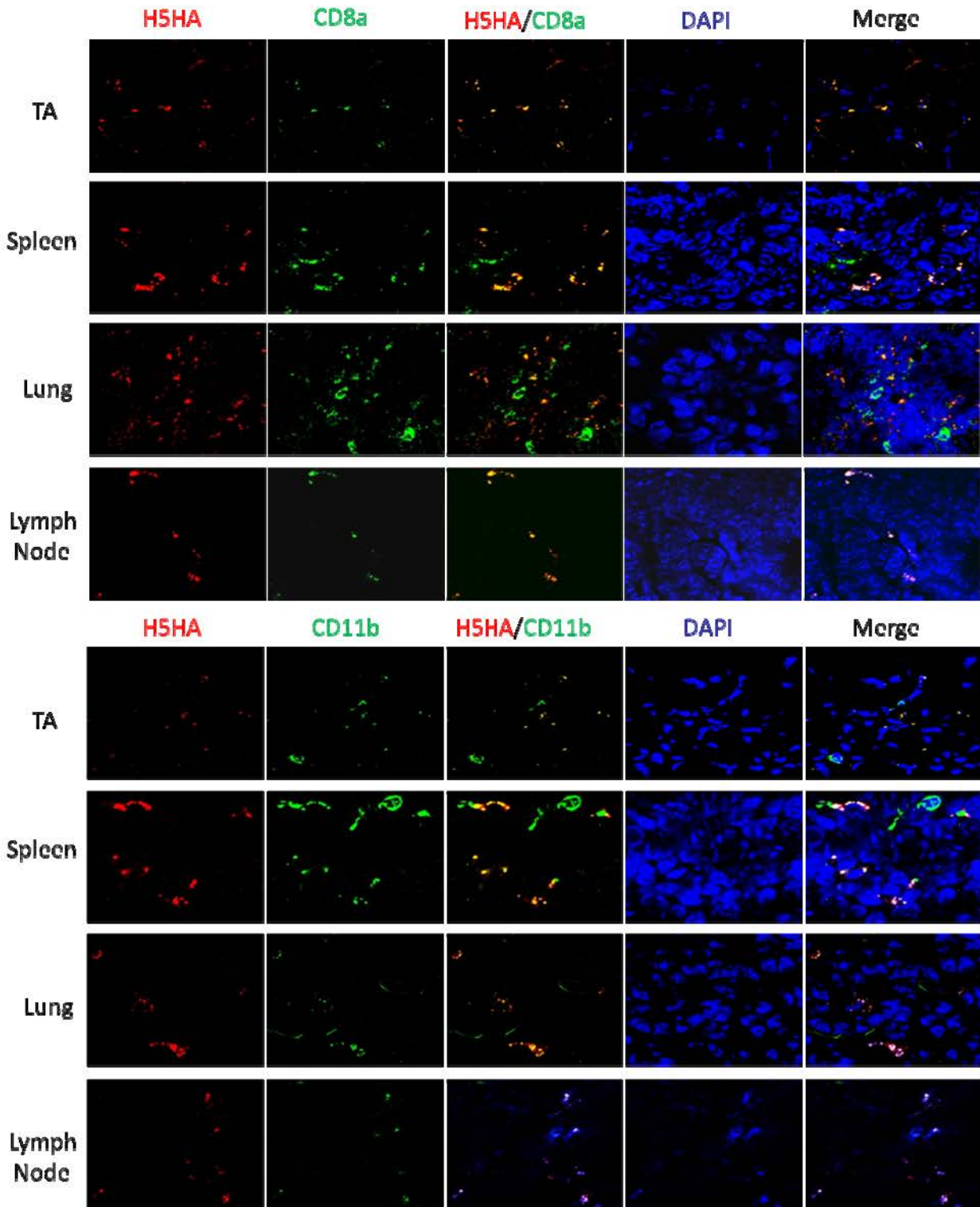
In the muscle tissues the H5HA gene was expressed in macrophages and T-cells after the administration of naked DNA or DNA/P85 or SP1017 in tibialis anterior muscles (**Figure 28, 29 and 30**). H5HA gene was also expressed in cytotoxic T-cells and T-helper cells in the muscles as well as in lungs, spleens and draining lymph nodes (**Figure 28, 29 and 30**). **Figure 31** presents the negative control specimens from PBS injected mice. Here we report that *H5HA gene expressed, co-localized in Antigen Presenting Cells (APC) such as macrophages and translocated to the distal sites*. These results demonstrated that in response to administration of DNA and DNA/P85 or SP1017 the APC incorporated, expressed and transported the H5HA transgene to the central immune organs.

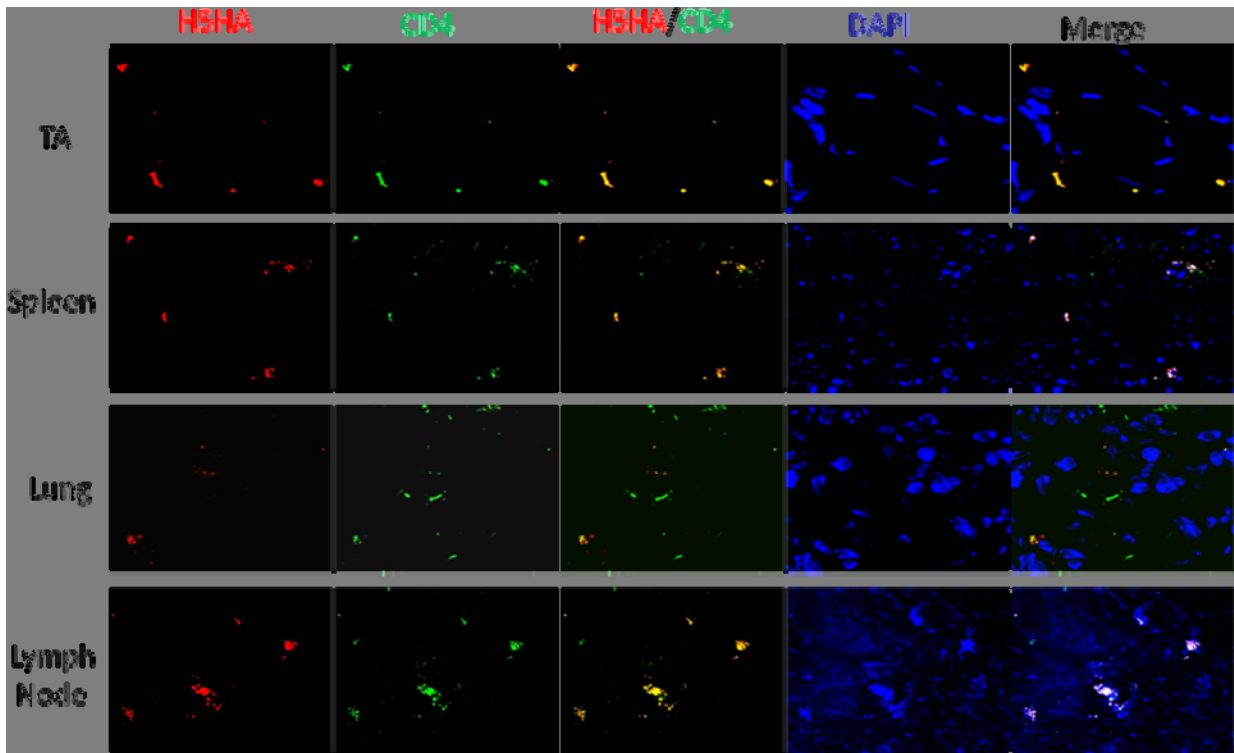






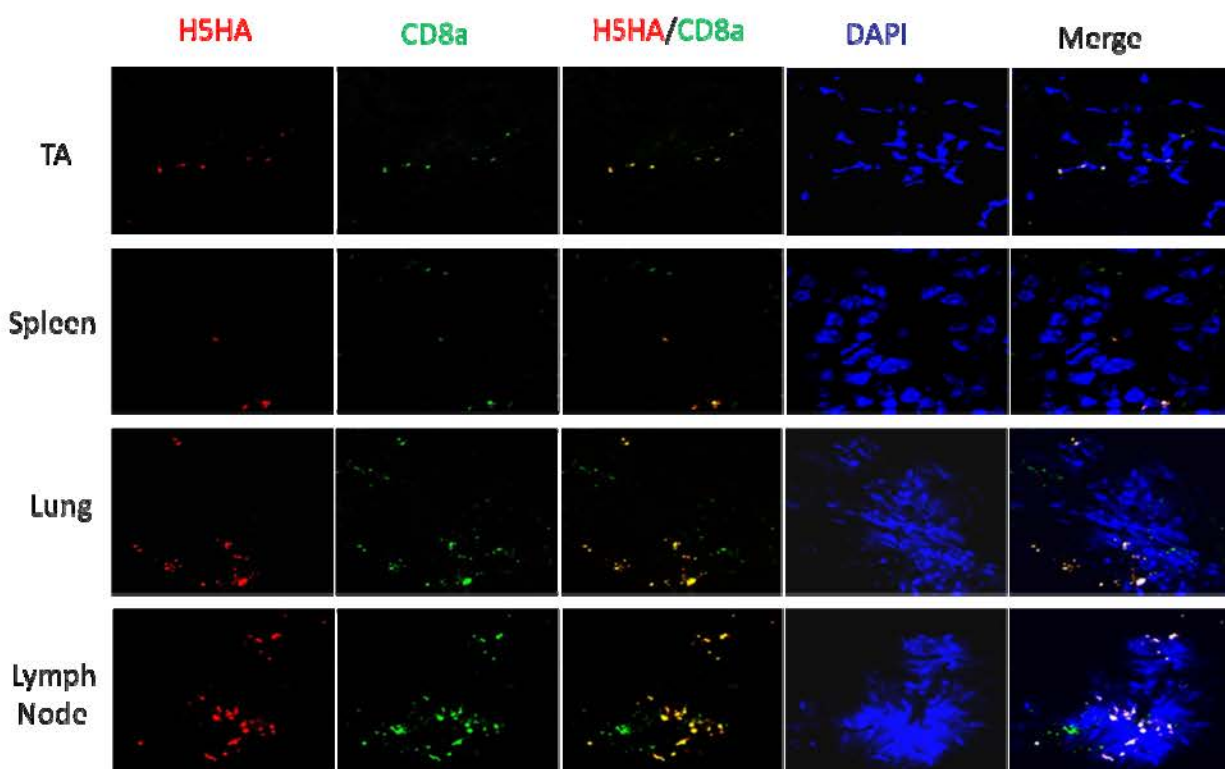
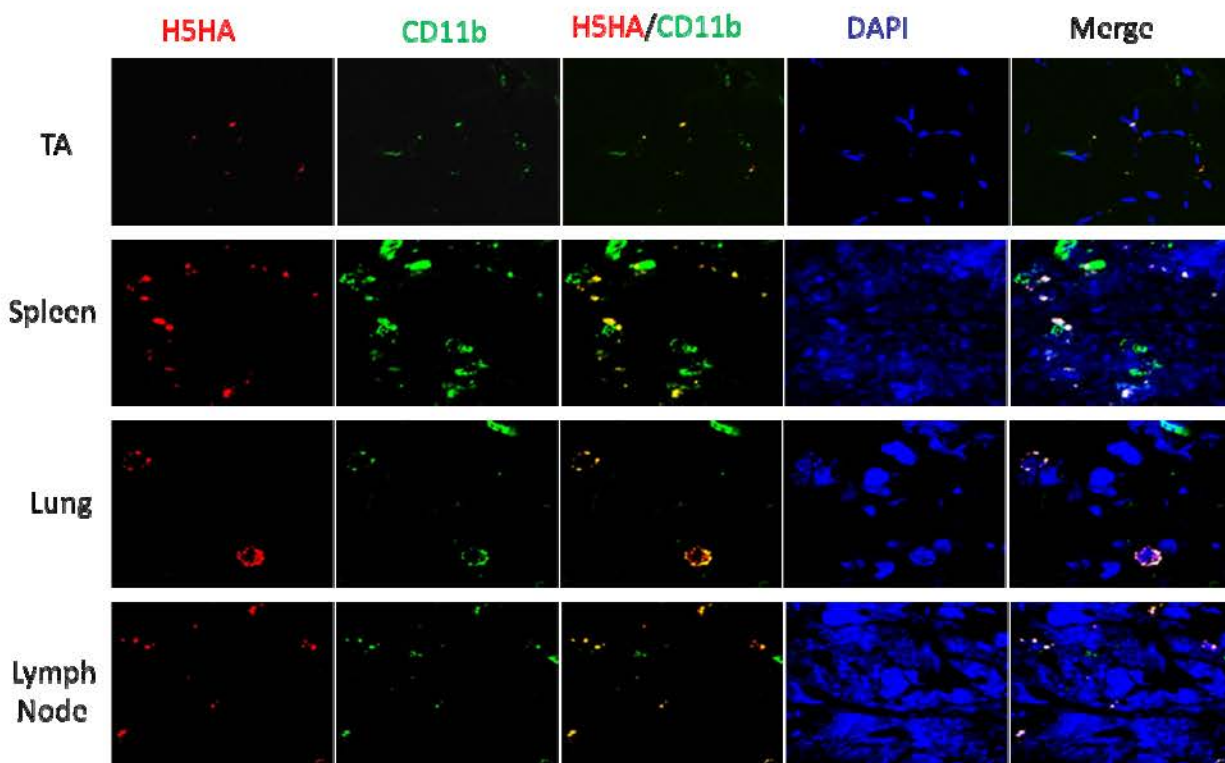
**Figure 28.** Confocal imaging of H5HA expression in macrophages (CD11b) and T-cells (CD8a and CD4) in muscle (Tibialis Anterior, TA), spleen, lung and draining lymph nodes after single *i.m.* injection of naked pH5HA DNA. The color staining corresponds to macrophages/T-cells (green), nucleus (blue) and H5HA (red). The 3<sup>rd</sup> and last panels in each row digitally superimposed to visualize the colocalization (yellow or white) Magnification 63x with 2x zoom.

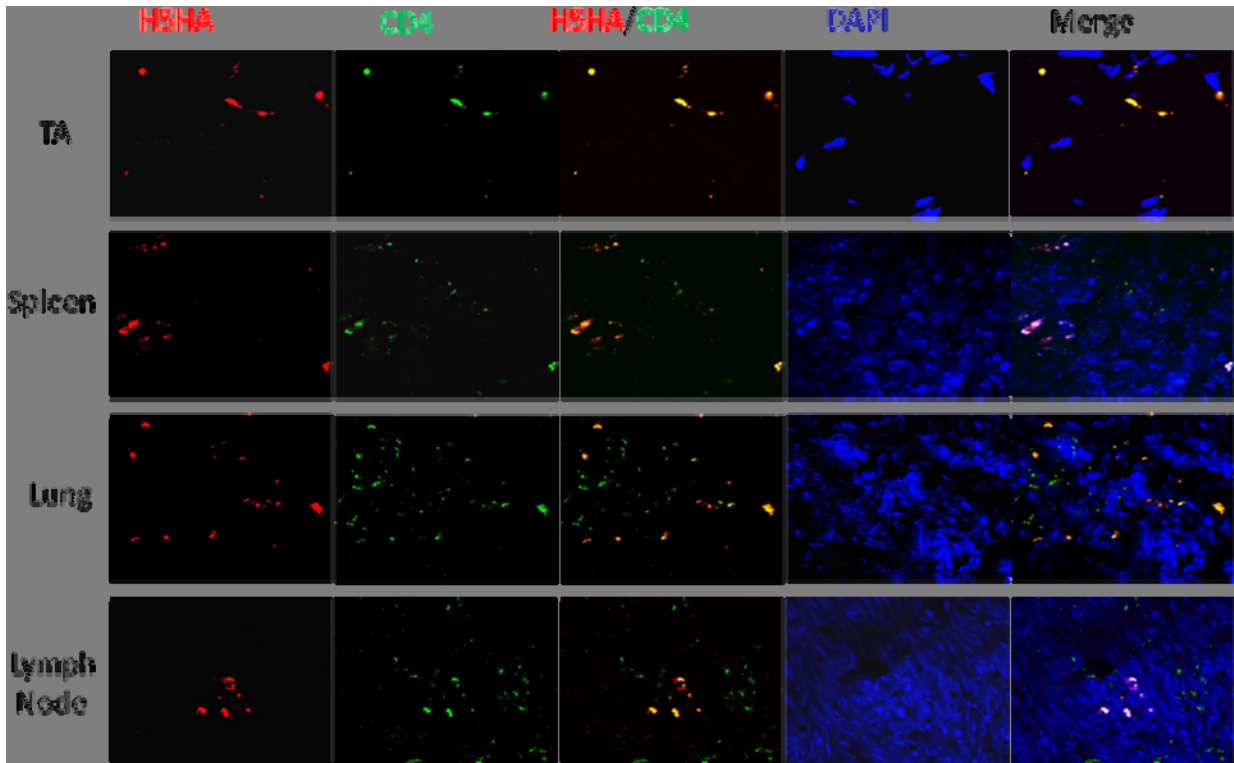




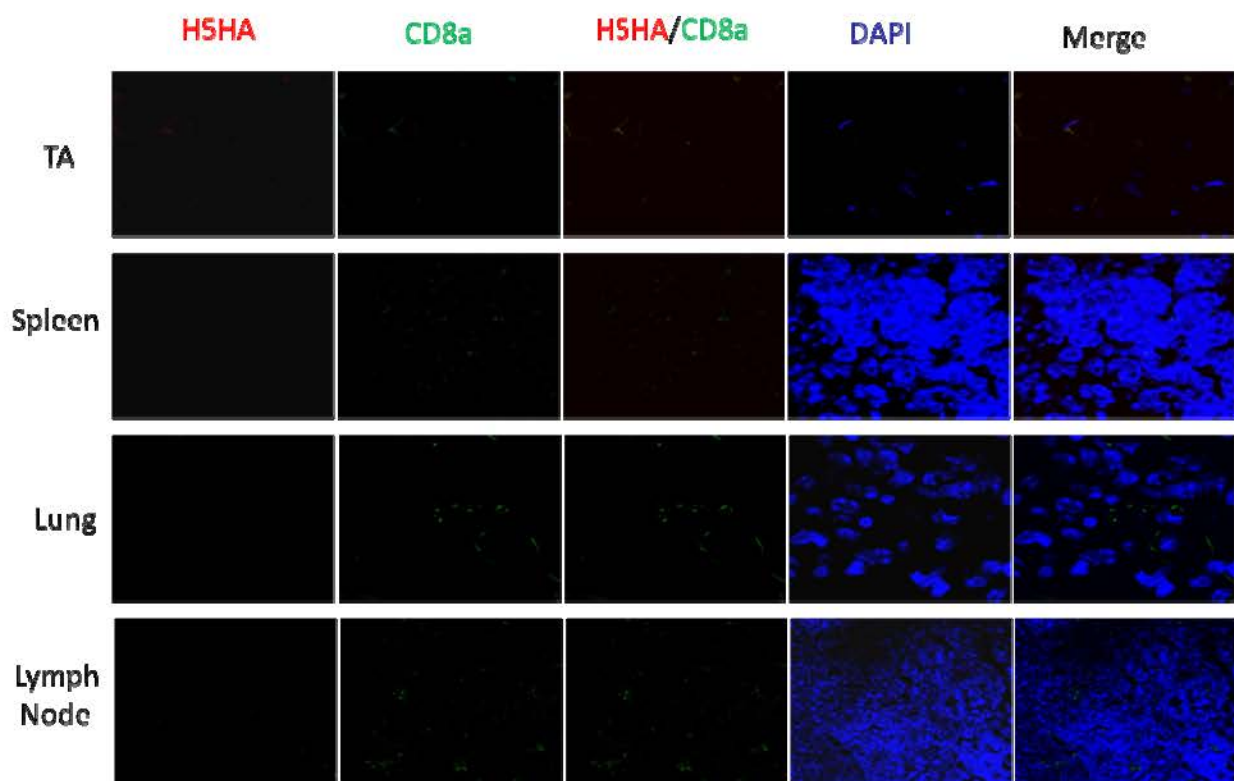
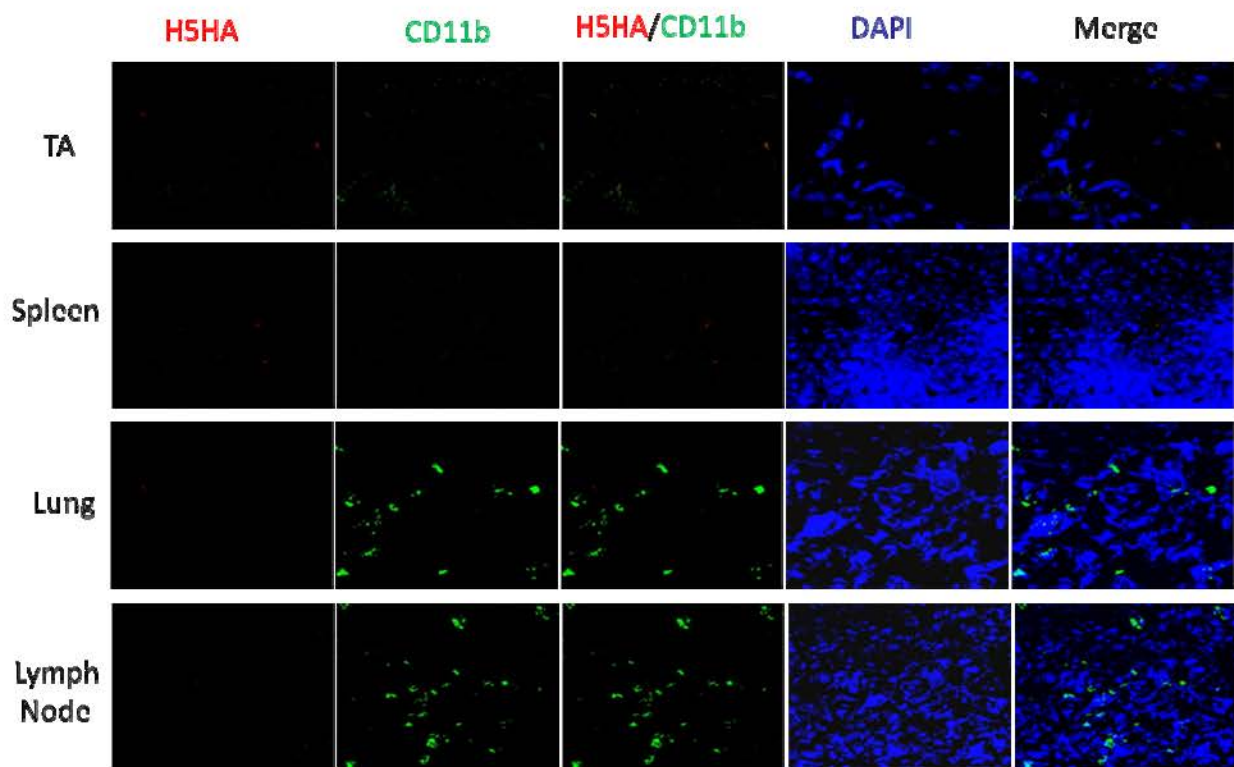
**Figure 29.** Confocal imaging of H5HA expression in macrophages (CD11b) and T-cells (CD8a and CD4) in muscle(Tibialis Anterior, TA), spleen, lung and draining lymph nodes after single *i.m.* injection of pH5HA +0.3% P85. The color staining corresponds to macrophages/T-cells (green), nucleus (blue) and H5HA (red). The 3<sup>rd</sup> and last panels in each row digitally superimposed to visualize the colocalization (yellow or white) Magnification 63x with 2x zoom.

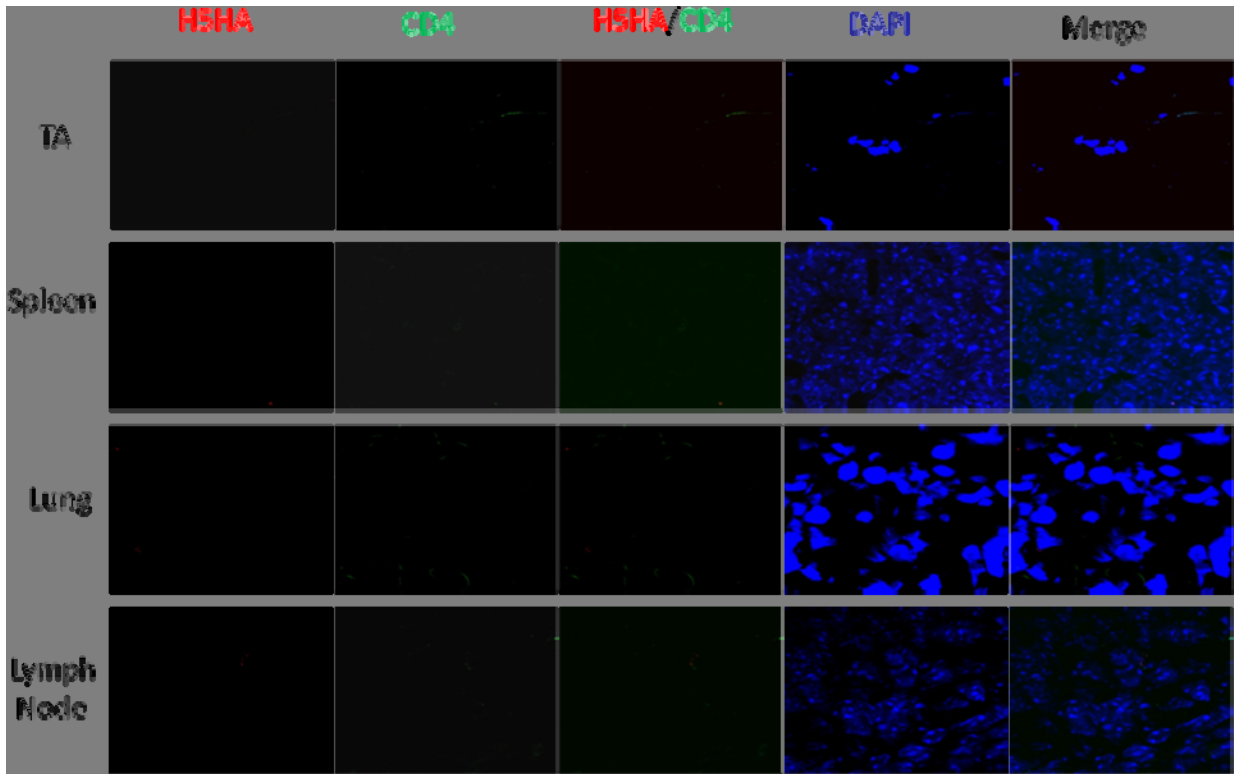






**Figure 30.** Confocal imaging of H5HA expression in macrophages (CD11b) and T-cells (CD8a and CD4) in muscle (Tibialis Anterior, TA), spleen, lung and draining lymph nodes after single *i.m.* injection of pH5HA +0.01% SP1017. The color staining corresponds to macrophages/T-cells (green), nucleus (blue) and H5HA (red). The 3<sup>rd</sup> and last panels in each row digitally superimposed to visualize the colocalization (yellow or white) Magnification 63x with 2x zoom.



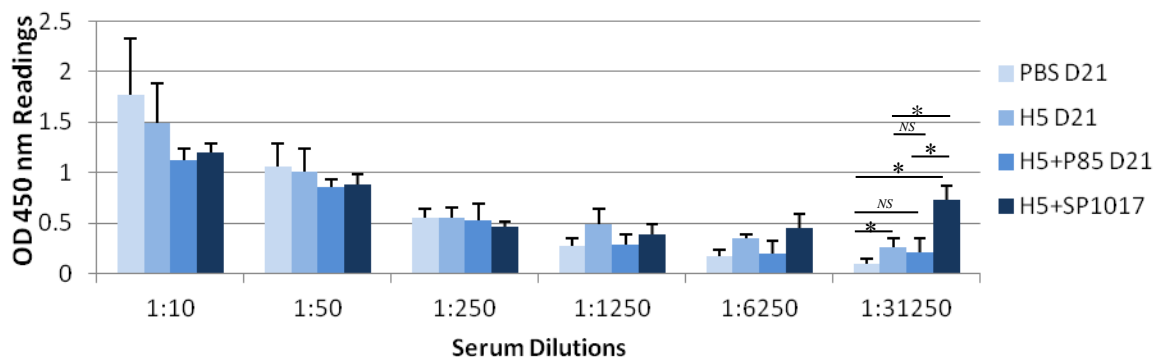


**Figure 31.** Confocal imaging of H5HA expression in macrophages (CD11b) and T-cells (CD8a and CD4) in muscle (Tibialis Anterior, TA), spleen, lung and draining lymph nodes after single *i.m.* injection of PBS. The color staining corresponds to macrophages/T-cells (green), nucleus (blue) and H5HA (red). The 3<sup>rd</sup> and last panels in each row digitally superimposed to visualize the colocalization (yellow or white) Magnification 63x with 2x zoom.

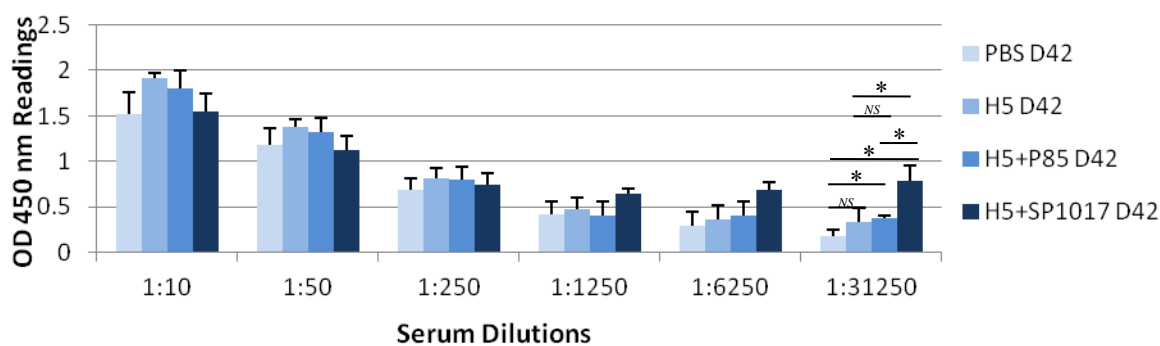
Next, we determined whether immunization with Pluronic/pVAX1-H5HA DNA vaccine could produce an immune response in a mouse. 50 µg of pVAX1-H5HA plasmid DNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered *i.m. in tibialis* anterior muscles in BALB/c mice (4 mice/group) and boosted on D29 with 50 µg of pVAX1-H5HA plasmid DNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017. Blood sera from individual mice were collected on day 21 (D21), 42 (D42) and 56 (D56). H5HA specific antibodies in blood sera were detected by enzyme-linked immunosorbent assay (ELISA) and our data shown in **Figure 32**. The present study demonstrated that pH5HA DNA vaccine with/without Pluronics could induce specific IgG antibodies in mice (**Figure 32**). All three DNA-based vaccine formulations were immunogenic and induced booster responses. However, the IgG antibody was detectable on the day 21 in the mice vaccinated with pH5HA DNA formulated with SP1017 and maintained the same high level until day 56 (**Figure 32 A, B, C and F**). There was no booster response observed in this group. Whereas, in the mice groups immunized with naked pH5HA DNA or pH5HA DNA with 0.3% w/v P85, a significant increase of specific IgG antibody levels were observed in blood serum on day 56 (**Figure 32A, B, C, D and E**). These data indicated that Pluronic/pVAX1-H5HA DNA vaccine formulations could induce a primary antibody response and that the level of the induced total IgG antibody was long lasting.

**A**

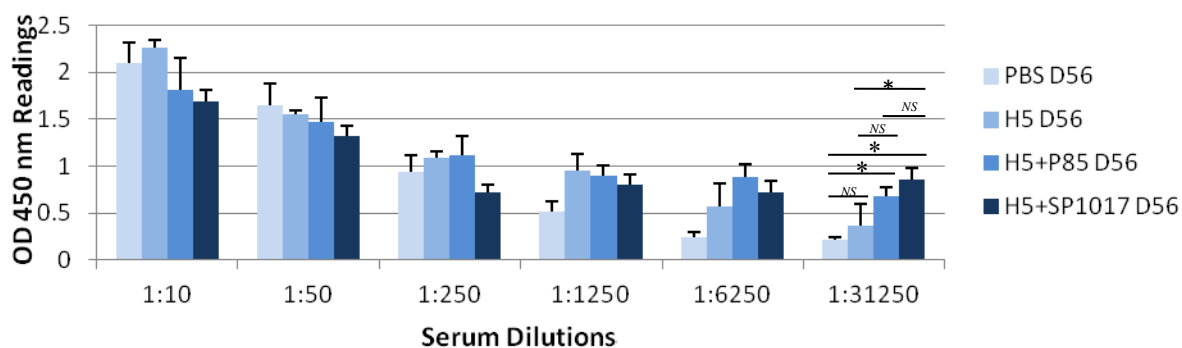
### anti-H5HA specific IgG Day 21

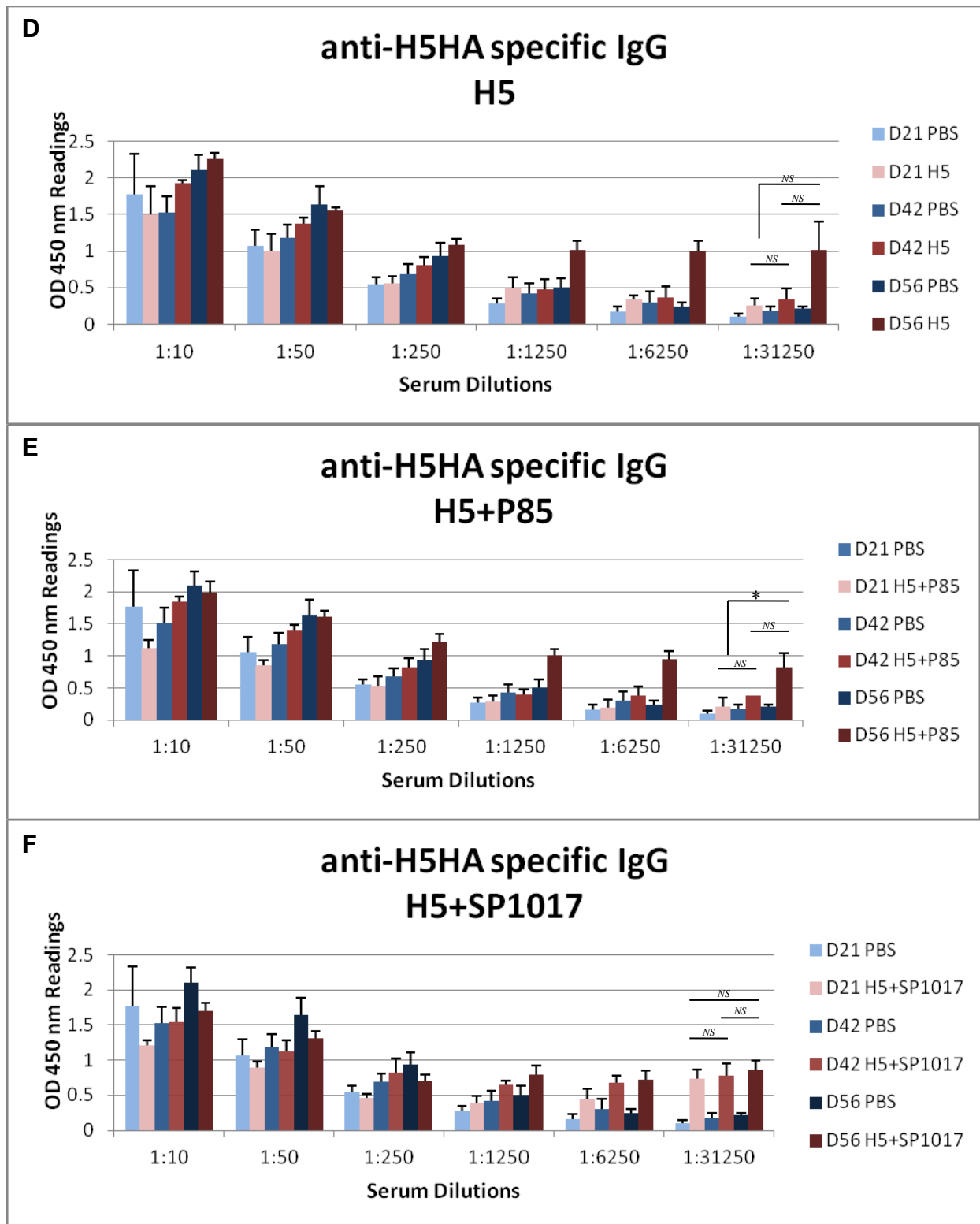
**B**

### anti-H5HA specific IgG Day 42

**C**

### anti-H5HA specific IgG Day 56





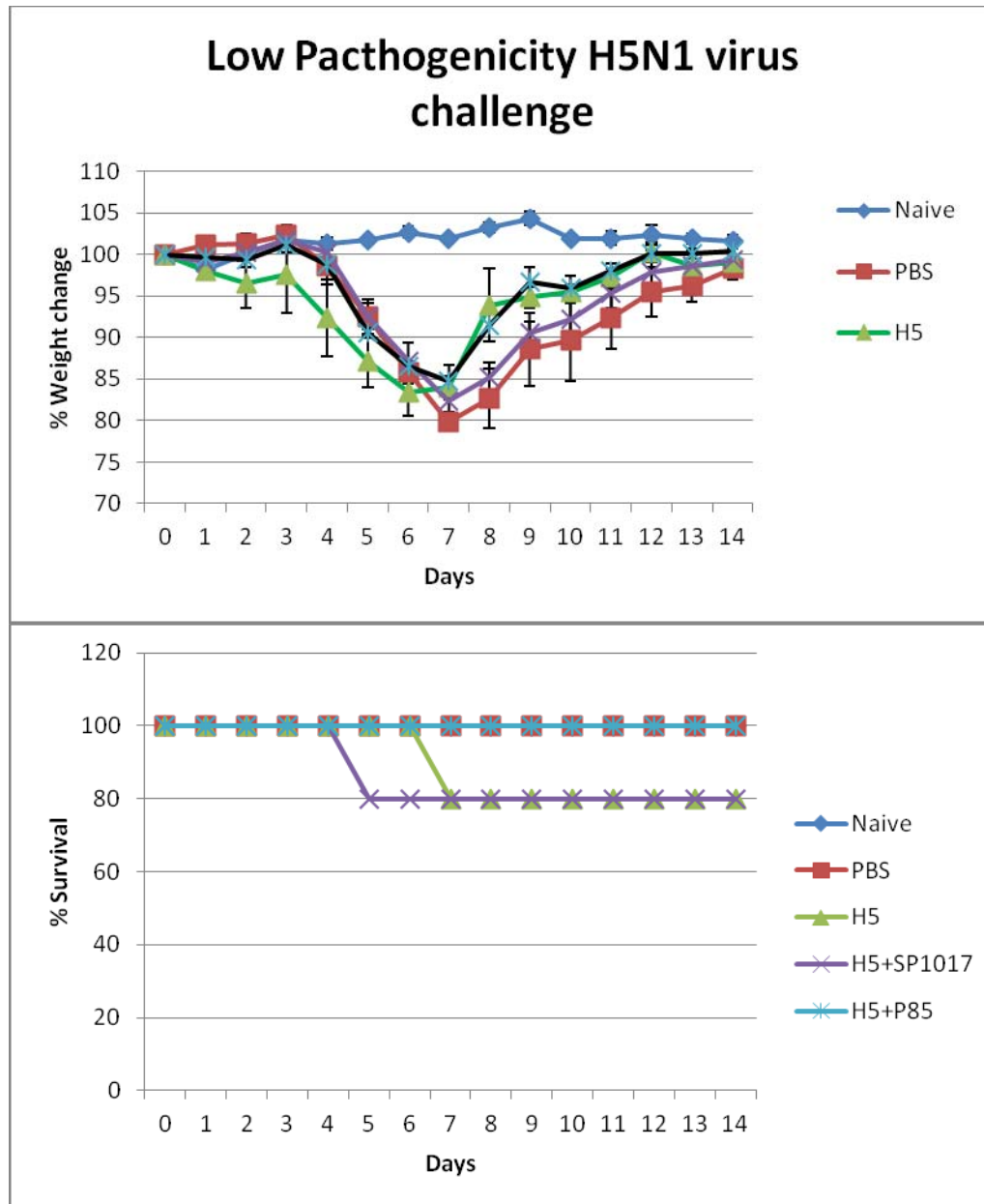
**Figure 32. Total serum IgG antibody responses to H5HA.** 50  $\mu$ g of pVAX1-H5HA pDNA formulated with/without 0.01% (w/v) SP1017 or 0.3% (w/v) P85 in 50  $\mu$ L of PBS were administered in tibialis anterior muscles of BALB/C mice (4 mice/group) and boosted with the same formulations on D29. Sera from individual mice were taken 21, 42 and 56 days after administration of vaccine formulations and



were tested for binding to H5 hemagglutinin by ELISA. Panel **A**, **B** and **C** are the H5HA specific IgG antibody titers by ELISA in sera at days 21, 42 and 56 (D21, D42 and D56) and panel **D**, **E** and **F** are the titers of H5HA specific IgG antibodies among different forms of vaccine. All data are presented as means  $\pm$  standard deviations and analyzed with Student's *t*-test ( $n=4$ ). \* $P<0.05$  and NS is not significant at 0.05 levels.

To achieve a milestone related to optimization of antibody response generated by DNA vaccine, we incorporated the boosting step into vaccination regimen. As was demonstrated in **Figure 32**, the boosting resulted in prolongation of antibody response generated by free HA DNA and its formulation with Pluronic P85. However, it is important to note that the boosting practically did not affect the level of antibody production after vaccination with HA DNA/SP1017 formulation. It was significantly higher on days 21 and 42 compared to other vaccine formulations studied. As a next step, we evaluated the efficacy of the identified vaccine formulations administered using boosting regimen. The goal of these experiments was to determine whether immunization with Pluronic/pVAX1-H5HA DNA vaccine can protect the mice against Low Pathogenicity H5N1 viral challenges. For these initial experiments on testing the protective efficacy of the vaccines mice viral challenge was administered via intranasal application. Dose of virus administered to the mice will be more accurately controlled using this methodology.

Vaccination of the animals was performed as follows: 50  $\mu$ g of pVAX1-H5HA plasmid DNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered i.m. in tibialis anterior muscles in BALB/c mice (5 mice/group) and boosted on day 29 (D29) with 50  $\mu$ g of pVAX1-H5HA plasmid DNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017. On day 63 after first vaccination the animals were challenged via intranasal administration of 30  $\mu$ l of neat  $1.9 \times 10^6$  TCID<sub>50</sub> Low pathogenicity influenza virus A/H5N1 (VNH5N1-PR8CDC-RG). The virus inoculums were obtained from Benchmark Biotech. The infected mice were observed daily for signs of illness or discomfort, and animal weight was measured for 14 days. All mice in the control group (PBS treatment) survived the challenges while exhibiting higher weight loss and delayed recovery compared to the vaccinated groups (**Figure 33A** and **B**). One of the mice from H5+SP1017 group suddenly died on day 4 post-challenge and another mouse from H5 group lost 30% of its original weight and had to be euthanized on day 6 (**Figure 33B**). All animals in H5+P85 vaccinated group survived the challenge study, and exhibited a weight loss of only 15% of their average pre-challenge body weight on day 7 and regained their original body weight (**Figure 33A** and **B**) on day 14. At the end point of the experiment mice were euthanized.

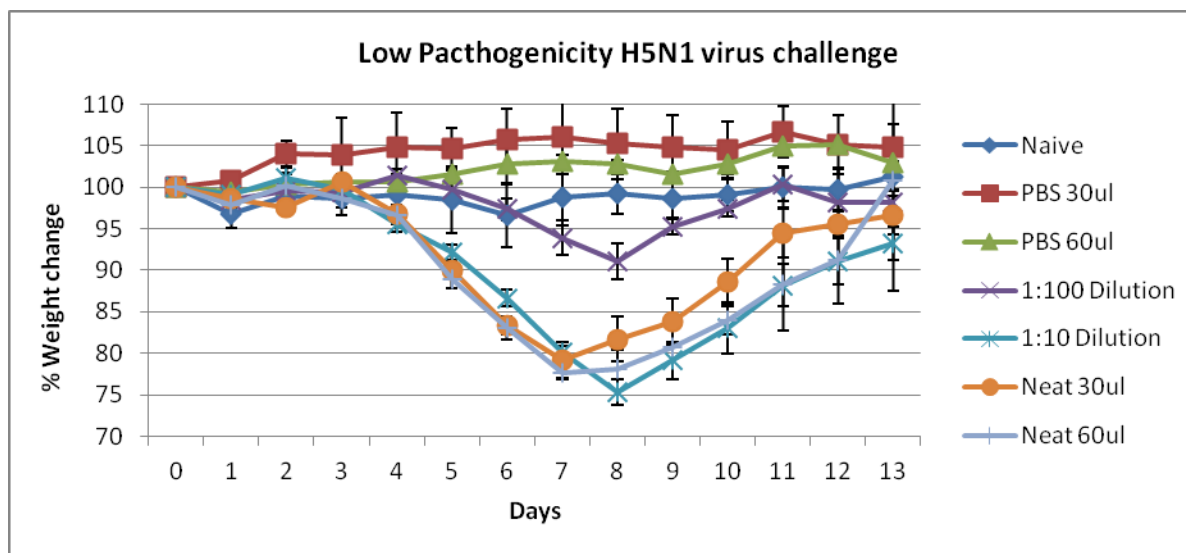


**Figure 33.** 50  $\mu$ g of pVAX1-H5HA pDNA formulated with/without 0.01% (w/v) SP1017 or 0.3% (w/v) P85 in 50  $\mu$ L of PBS were administered in tibialis anterior muscles of BALB/c mice (5 mice/group) and boosted with the same formulations on D29. Mice were challenged intranasally on D63 with 30  $\mu$ L of neat  $1.9 \times 10^6$  TCID<sub>50</sub> Low pathogenicity influenza virus A/H5N1 (VNH5N1-PR8CDC-RG). (A) body weight changes (data expressed as a percentage of the pre-challenged body weight and presented as mean  $\pm$  SEM,  $n=3-5$ ) and (B) animal survival was daily monitored for 14 days (Day 0 = day virus challenged).

To determine the minimum amount of virus that would give a 100% mouse infectious dose (MID<sub>100</sub>) of the low pathogenic Influenza virus A/H5N1 (VNH5N1-PR8CDC-RG), dilutions of neat  $1.9 \times 10^6$  TCID<sub>50</sub> of 1:10 and 1:100 were made in sterile PBS. PBS and undiluted virus were used as controls. Either 30 or 60 microliters were instilled in the nares of BALB/c mice and

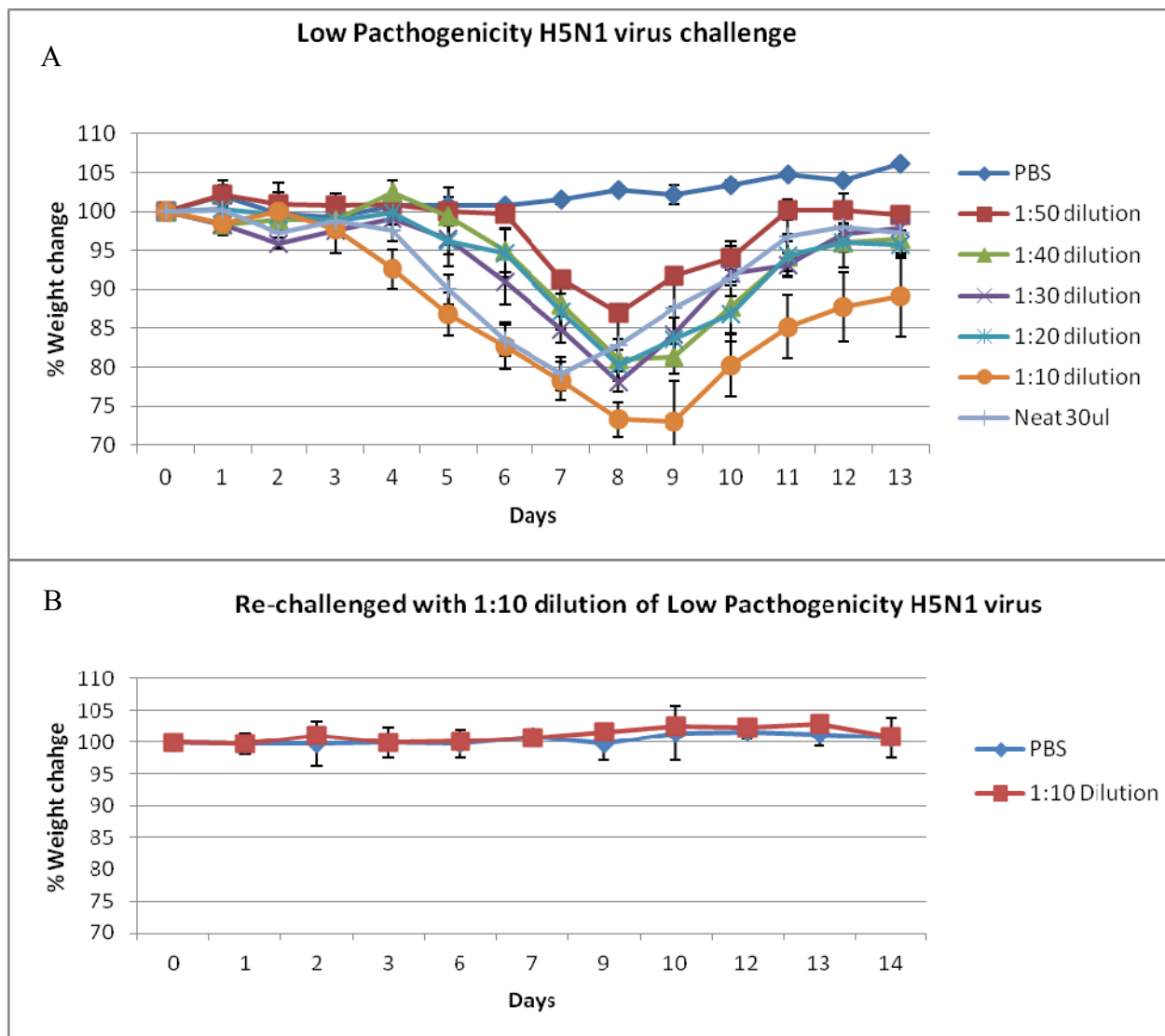


weight loss/gain was observed for 13 days. Three (3) mice were used per treatment group and two (2) mice were used in the control (PBS) group. All mice, excluding both sets of PBS controls and the Naive mice showed marked weight loss by day 8, but all mice recovered and body weight was increasing to near starting weight by day 13. It was determined that further work was necessary and that viral dilutions between 1:10 and 1:100 in PBS should be done to further define the  $MID_{100}$  of the virus stock (**Figure 34**).



**Figure 34.** BALB/c mice were challenged intranasally with 30  $\mu$ l of neat  $1.9 \times 10^6$  TCID<sub>50</sub> and diluted Low pathogenicity influenza virus A/H5N1 (VNH5N1-PR8CDC-RG). Body weight changes (data expressed as a percentage of the pre-challenged body weight and presented as mean  $\pm$  SEM,  $n = 2-3$ ) and monitored for 13 days (Day 0=day virus challenged).

To better determine the 100 % mouse infectious dose ( $MID_{100}$ ) of the  $1.9 \times 10^6$  TCID<sub>50</sub> low pathogenic Influenza virus A/H5N1 (VNH5N1-PR8CDC-RG), dilutions of 1:10, 1:20, 1:30, 1:40, and 1:50 were made in PBS. PBS and undiluted virus were used as controls. 30 microliters were instilled in the nares of BALB/c mice and weight loss/gain was observed for 13 days. Three (3) mice were used per treatment group and two (2) mice were used in the control (PBS) group. All mice, excluding the PBS controls showed marked weight loss by day 8, but all mice recovered and body weight was increasing to near starting weight by day 13. The Influenza standard for infection is weight loss below 80% of the original body weight. *It was determined that a 1:10 dilution in PBS of the virus stock was the minimum  $MID_{100}$  as this was the only dilution that achieved 100% of mice with weight loss below 80%; this dilution will be used for further experiments (Figure 35A).* The 1:10 virus challenged mice and the PBS control group from these experiments (**Figure 35A**), were re-challenged with a 1:10 virus dilution (**Figure 35 B**) and observed for 14 days. All animals in this re-challenged group survived the challenge study, and exhibited no change of body weight as PBS controlled group (**Figure 35B**). This result demonstrated that mice challenged with 1:10 dilution of  $1.9 \times 10^6$  TCID<sub>50</sub> low pathogenic Influenza virus A/H5N1 (VNH5N1-PR8CDC-RG) protected the mice against the influenza.



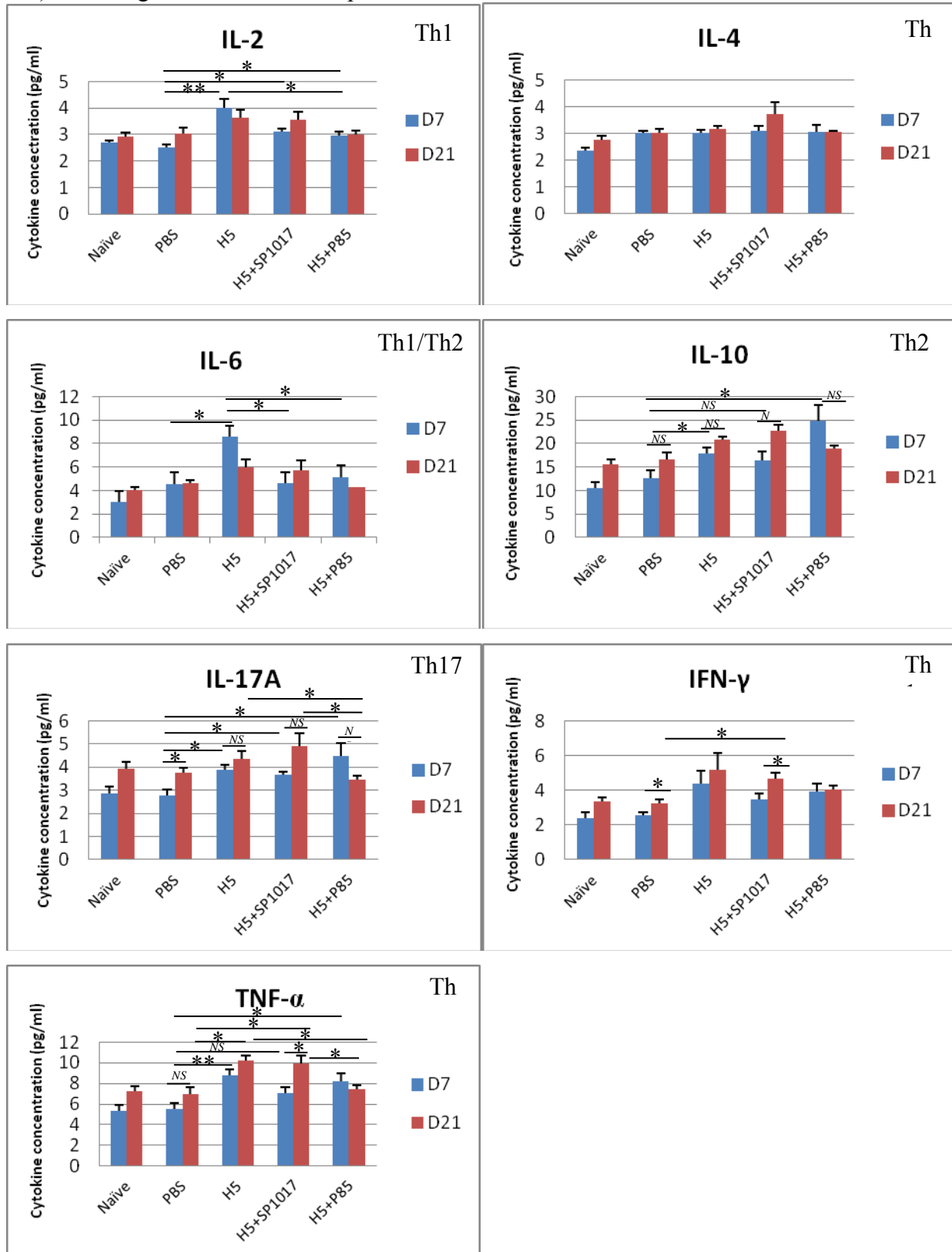
**Figure 35.** A. BALB/c mice were challenged intranasally with 30  $\mu$ l of neat  $1.9 \times 10^6$  TCID<sub>50</sub> and diluted Low pathogenicity influenza virus A/H5N1 (VNH5N1-PR8CDC-RG). Body weight changes (data expressed as a percentage of the pre-challenged body weight and presented as mean  $\pm$  SEM,  $n = 2-3$ ) and monitored for 13 days (Day 0=day virus challenged). B. PBS and 1:10 dilution group of mice were re-challenged with the same formulations on day 28 and body weight changes were monitored for 14 day.

## Year 4

### Cytokine responses

Cytokines serve as couriers between cells of the immune system and play a crucial role in orchestrating the immune response after vaccination. Therefore, they are an important indicator of effective induction of immune effectors. In order to determine if our Pluronic/pVAX1-H5HA DNA vaccine formulations induce any cytokine responses, in this current study we measured the concentrations of Th1 (IL-2 and INF- $\gamma$ ), Th2 (IL-4 and IL-10) and Th17 (IL-17) cytokines in sera from mice vaccinated with 100  $\mu$ g of pVAX1-H5HA pDNA formulated with/without 0.01% (w/v) SP1017 or 0.3% (w/v) P85 on day 7 and 21. Th1/Th2/Th17 cytokine concentrations were

determined using the Mouse Cytometric Bead Array (CBA) Kit (BD Biosciences, San Diego, CA) following the manufacturer's protocol.

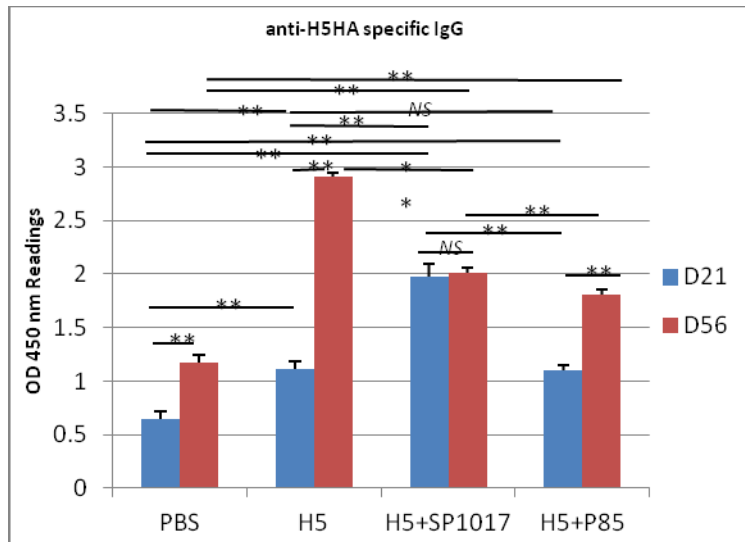


**Figure 36.** The cytokine response induced in the blood serum after pH5HA vaccination. 100 µg of pVAX1-H5HA pDNA formulated with/without 0.01% (w/v) SP1017 or 0.3% (w/v) P85 in 50 µL of PBS were administered in tibialis anterior muscles of BALB/c mice (4 mice/group) and boosted with the same formulations on D28. Sera from individual mice were taken 7 and 21 days after administration of vaccine formulations and were used to measure Th1 (IL-2, TNF and IFN-g), Th2 (IL-4 and IL-10), Th1/Th2 (IL-6) and Th17 (IL-17) cytokines by Mouse Cytometric Bead Array (CBA). The data is presented as the mean cytokine concentration (pg/ml)±SEM and analyzed with Student's *t*-test (n=4). \**P*<0.05, \*\**P*<0.01 and NS is not significant at 0.05 levels.

Pluronic /pVAX1-H5HA pDNA formulated vaccine groups had significantly higher levels of Th1 (IL-2 and TNF), Th2 (IL-10) and Th17 (IL-17) cytokines on day 7 compared to PBS injected mice (**Figure 36**). However, on day 21, only sera from H5+SP1017 vaccinated mice induced significant increase in TNF and IFN-g (Th1) cytokines. Our data also showed strong Th17 responses. On the other hand, IM vaccination induced significantly higher concentrations of IL-10 (Th2) in H5+P85 injected group of mice on day 7 as compared to other groups but, there was no IL-4 cytokine responses in any of the vaccinated groups. However, only pDNA vaccinated group showed significant increase of IL-6 (Th1/Th2) cytokines on day 7 (**Figure 36**). These results demonstrated that Pluronic /pVAX1-H5HA pDNA vaccine formulations induced Th1, Th2 and Th17 cytokine responses in mice.

#### **H5HA specific antibody responses**

In order to determine if our Pluronic/pVAX1-H5HA DNA vaccine formulations induce antibody responses we performed the ELISA and our data shown in **Figure 37**. The present study demonstrated that pH5HA vaccine with/without Pluronic could induce specific IgG antibodies in mice (**Figure 37**). On the day 21 the IgG antibody level in pH5HA /SP1017 treated groups was higher than those in the groups received the naked pH5HA or pH5HA formulated with 0.3% w/v P85 (**Figure 37**). However, on the day 56 the naked pH5HA exhibited significant increase in IgG antibody compared to pH5HA with Pluronic SP1017 or P85. The mice vaccinated with pH5HA/SP1017 did not show any booster response while the naked pH5HA or pH5HA formulated with 0.3% w/v P85 showed significant booster effect (**Figure 37**). These data indicated that Pluronic/pVAX1-H5HA DNA vaccine formulations could induce lasting primary antibody responses.

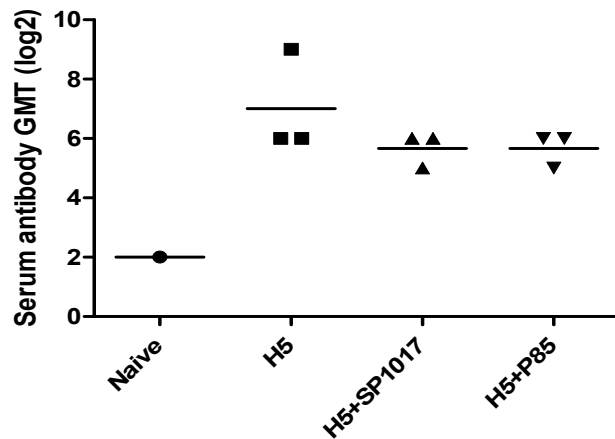


**Figure 37.** Total serum IgG antibody responses to H5HA. 100 µg of pVAX1-H5HA pDNA formulated with/without 0.01% (w/v) SP1017 or 0.3% (w/v) P85 in 50 µL of PBS were administered in tibialis anterior muscles of BALB/c mice (4 mice/group) and boosted with the same formulations on D28. Sera from individual mice were taken 21 and 56 days after administration of vaccine formulations and were tested for binding to H5 hemagglutinin by ELISA. All data are presented as means  $\pm$  SEM and analyzed with Student's *t*-test ( $n=4$ ) at 1:31250 serum dilution. \*\* $P<0.01$ , \* $P<0.05$  and NS is not significant at 0.05 levels.

### Hemagglutination inhibition assay

The hemagglutination inhibition (HAI) assay was adapted from the CDC laboratory-based influenza surveillance manual (5). To inactivate non-specific inhibitors, sera were treated with receptor destroying enzyme (RDE) (Denka-Seiken, Tokyo, Japan) prior to being tested. Briefly, three parts RDE was added to one part sera and incubated overnight at 37°C. RDE was inactivated by incubation at 56°C for ~60 min. RDE-treated sera were two-fold serially diluted in v-bottom microtiter plates. An equal volume of virus iVN04 (NR-4143, Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH, Bethesda, MD), adjusted to approximately 4 HAU/25 µl was added to each well. The plates were covered and incubated at room temperature for 30 min followed by the addition of 1 % turkey erythrocytes (TRBC) (Innovative Research, MI, USA). The plates were mixed by agitation, covered, and the TRBC were allowed to settle for 60 min at room temperature. The HAI titer was determined by the reciprocal dilution of the last well, which contained non-agglutinated TRBC. Positive and negative serum controls were included for each plate.

Mice received 2 doses of pH5HA had higher serum HAI Ab titers against the A/Vietnam/1203/2004 (H5N1) virus compared to naive mice (**Figure 38**). A very similar level of serum HAI Ab titers were observed in the groups of mice those primed and boosted with pH5HA/0.01% (w/v) SP1017 or 0.3% (w/v) P85 (**Figure 38**).



**Figure 38.** The hemagglutination inhibition (HAI) antibody response induced after pH5HA vaccination. 100  $\mu$ g of pVAX1-H5HA pDNA formulated with/without 0.01% (w/v) SP1017 or 0.3% (w/v) P85 in 50  $\mu$ L of PBS were administered in tibialis anterior muscles of BALB/c mice (3 mice/group) and boosted with the same formulations on D28. Sera from individual mice were taken 56 days after administration of vaccine formulations and were used to measure HAI antibody response against A/Vietnam/1203/2004 (H5N1). The geometric mean titer obtained from 3 mice  $\pm$ SD.

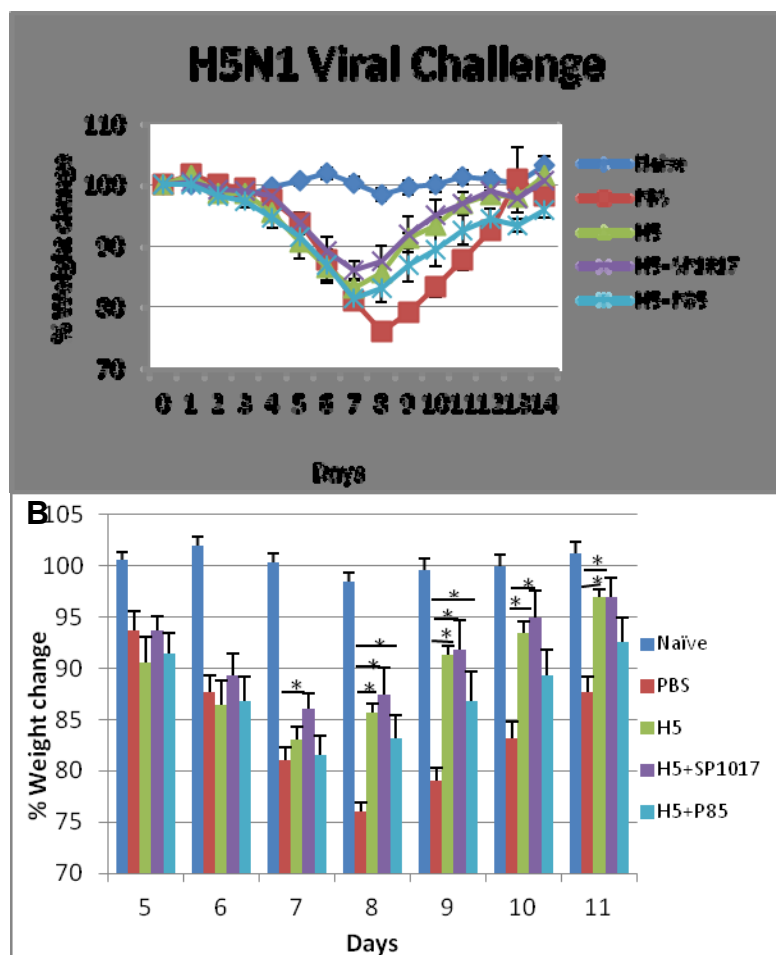
### Viral challenge study

Next, we determined whether immunization with Pluronic/pVAX1-H5HA DNA vaccine formulations can protect the mice against Low Pathogenicity H5N1 viral challenges.

100  $\mu$ g of pVAX1-H5HA plasmid DNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered i.m. in tibialis anterior muscles in BALB/c mice (9 mice/group) and boosted on day 28 (D28) with the same formulations. On day 64 after the first vaccination the animals were challenged via intranasal administration of 30  $\mu$ L of 1:10 diluted  $1.9 \times 10^6$  TCID<sub>50</sub> Low pathogenicity influenza virus A/H5N1 (VNH5N1-PR8CDC-RG). Four mice from each group were euthanized on day 3 for determination of viral load in lung while the remaining 5 mice in each group were monitored for development of clinical signs and loss of body weight for 14 days (**Figure 39**).

All mice in the control group (PBS treatment) survived the challenges while exhibiting higher weight loss and delayed recovery compared to the vaccinated groups (**Figure 39A** and **B**). All the animals in Pluronic/pVAX1-H5HA DNA vaccinated groups showed less discomfort. H5+SP1017, H5+P85 and pDNA alone vaccinated group of animals exhibited a weight loss of only 14%, 19% and 17% respectively of their average pre-challenge body weight on day 7 and regained their original body weight on day 14 (**Figure 39A**). Though, Pluronic/pVAX1-H5HA DNA vaccine formulation significantly protected the weight loss compared to PBS control group, there was no significant difference in weight loss observed among the Pluronic/pVAX1-H5HA DNA vaccinated groups (**Figure 39B**).

A

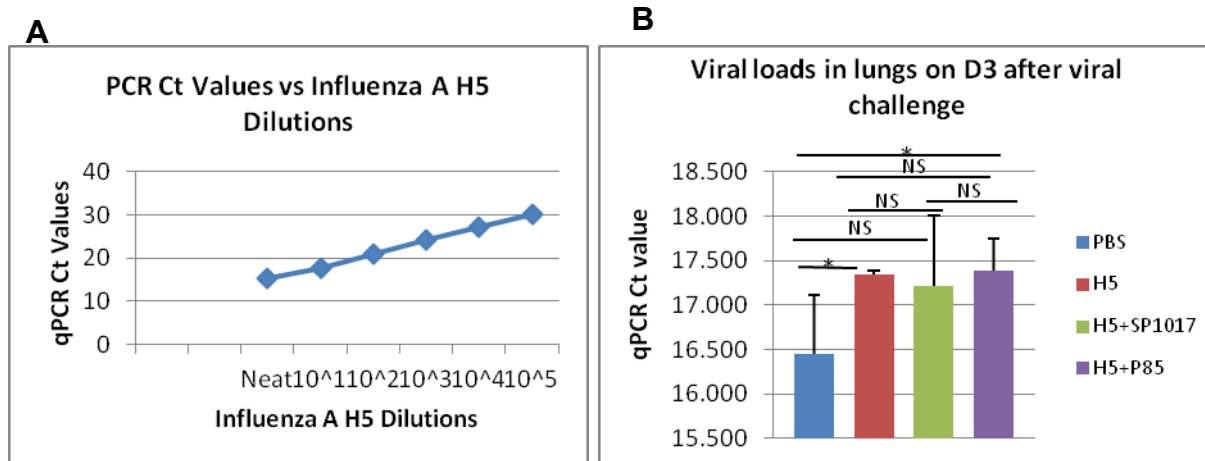


**Figure 39.** 100  $\mu$ g of pVAX1-H5HA pDNA formulated with/without 0.01% (w/v) SP1017 or 0.3% (w/v) P85 in 50  $\mu$ l of PBS were administered in tibialis anterior muscles of BALB/c mice (5 mice/group) and boosted with the same formulations on D28. Mice were challenged intranasally on D64 with 30  $\mu$ l of 1:10 diluted  $1.9 \times 10^6$  TCID<sub>50</sub> Low pathogenicity influenza virus A/H5N1 (VNH5N1-PR8CDC-RG). A, Body weight changes data expressed as a percentage of the pre-challenged body weight and presented as mean  $\pm$  SEM ( $n = 3-5$ ) and B, data analyzed with Student's  $t$ -test ( $n=5$ ), \* $P < 0.05$ .

In order to determine the viral load in the challenged mice lungs, lung tissues were collected from the vaccinated groups of mice on 3 days post-challenge. Lung tissues were then tested by Real-time RT-PCR to determine viral load in each group of mice. Lung tissues were processed and analyzed following the modified procedure of Alsharifi, et. al., 2009 (6). PCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR System. For the standard curve, normal, non-influenza challenged mice lungs (PBS controls) were used. RNA from Stock influenza A H5 virus was extracted using the Qiagen QiAmp Viral RNA Kit Mini Kit (Qiagen, Carlsbad, CA). For the standard curve, the H5 extracted viral RNA were standardized to the normal mouse lungs. Standard curves were performed with each set of PCR reactions (**Figure 40 A**).

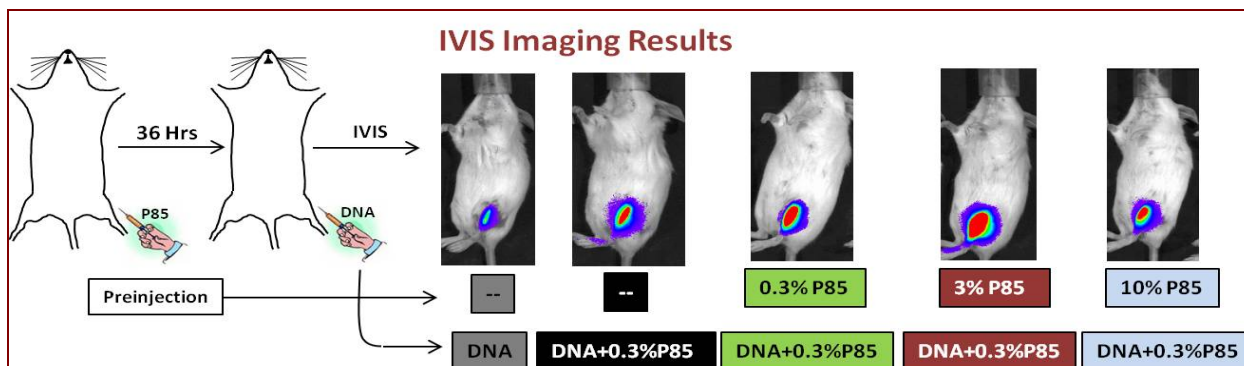
Quantitation of viral genetic loads in lungs of Pluronic/pVAX1-H5HA DNA vaccinated groups demonstrated protective effect of the vaccine formulations compared to PBS controlled mice on day 3 post-viral challenge (**Figure 40**). Pluronic P85/ pVAX1-H5HA DNA vaccinated groups had significantly less viral RNA in the lungs compared to PBS vaccinated mice. (**Figure 40B**). However, viral loads determined for Pluronic/pVAX1-H5HA DNA vaccinated groups did not show any significant differences among them.

Overall, these results demonstrated that Pluronic/pVAX1-H5HA DNA vaccine formulations have significant protective effective over PBS control treatment against Low pathogenicity influenza virus A/H5N1 (VNH5N1-PR8CDC-RG).



**Figure 40.** Viral loads in lungs on day 3 post-viral challenge. 100  $\mu$ g of pVAX1-H5HA pDNA formulated with/without 0.01% (w/v) SP1017 or 0.3% (w/v) P85 in 50  $\mu$ l of PBS were administered in tibialis anterior muscles of BALB/c mice (4 mice/group) and boosted with the same formulations on D28. Mice were challenged intranasally on D64 with 30  $\mu$ l of 1:10 diluted  $1.9 \times 10^6$  TCID<sub>50</sub> Low pathogenicity influenza virus A/H5N1 (VNH5N1-PR8CDC-RG). **A.** Threshold (Ct) values for H5 standard control dilutions. **B.** Viral Loads in lungs on D3 post-viral challenge as expressed mean qPCR Ct values  $\pm$  SD and data analyzed with Student's *t*-test (n=4). \**P*<0.05, NS is not significant at 0.05 levels.

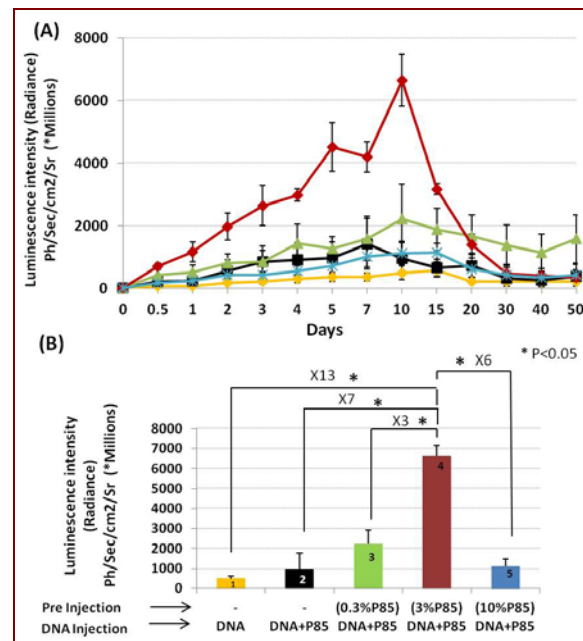
We also continued studies in the task aimed to maximize levels and duration of transgene expression after administration in skeletal muscle in a mouse. Previous studies with Pluronic P85 mediated *in vivo* gene delivery have reported increase in reporter protein expression up to an order of magnitude when P85 is co-delivered with plasmid DNA (pDNA) compared to pDNA alone in healthy BALB/c mice (7). This increase in gene expression was also found in distal lymphoid organs (draining lymph nodes and spleen) in addition to injected muscle. Our rationale here was to study the effect of role of immune cells in Pluronic mediated increase in transgene expression. We performed Pluronic formulated pDNA injections in bilateral tibialis anterior muscles preinjected with increasing concentrations of P85 i.e. local inflammation model to study the role played by immune cells in increasing the transgene expression by increasing the local inflammation (**Figure 41**).



**Figure 41.** Scheme of experiment and representative image of non-invasive *in vivo* imaging (Xenogen IVIS 300) results. Animals were preinjected with increasing concentrations of P85 and then injected DNA 36 hours later. (n=4)



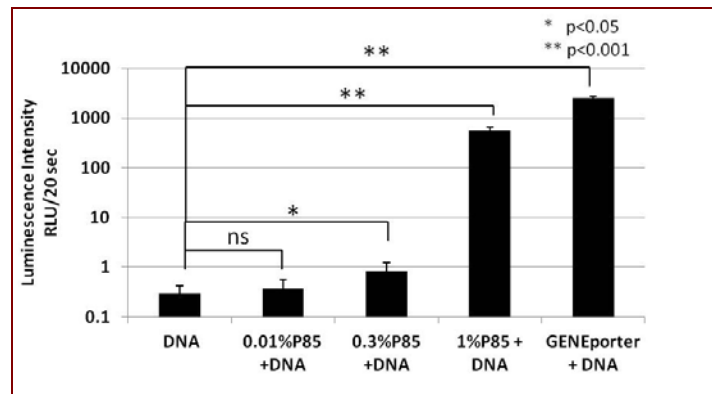
To prove our hypothesis we induced local infiltration of immune cells in bilateral tibialis anterior muscle of BALB/c mice by injecting increasing dose of P85 (0.3 %, 3% and 10%) in 50 ul of PBS (preinjection model) followed by 10 $\mu$ g DNA+P85 (0.3%) injections at 36 hour (i.e. estimated neutrophil infiltration peak) after pre-injection. DNA/DNA+P85 injections without preinjections were used as controls for preinjection model. Luciferase reporter protein expression was monitored by injecting D-luciferin (150 mg luciferin/kg body weight) *i.p.* followed by non invasive *in vivo* bioluminescent imaging (exposure for 5 seconds) using Xenogen IVIS 200 imaging system. Kinetics of protein expression was monitored till day 50 post DNA injection with a peak of luciferase expression at day 10 (**Figure 42A**). Quantification of images at D10 demonstrated that animals preinjected with 3% P85 expressed luciferase activity 13 and 7 times compared to DNA alone and DNA+P85 respectively (**Figure 42B**). Infiltration of immune cells (e.g. neutrophils, macrophages etc) have long been correlated with repair and regeneration of muscle tissues (8). Increase in gene expression with increasing P85 (0.03% and 3%) could be explained by increased immune cell infiltration and their role in increasing the transgene expression and tissue repair. On the contrary 10% P85 preinjection may induce tissue injury beyond repair which could be the reason for decreased gene expression.



**Figure 42.** Effect of P85 preinjections on gene expression after single i.m. injection of 10  $\mu$ g plasmid DNA in 50 mL PBS (**A**). Quantitative data of *in vivo* imaging study at day 10 (**B**). Data are mean  $\pm$  SEM (n =4). P values were obtained by the means of Student's t test.

We further attempted to understand the role of P85 in increasing the transgene expression in immune cells by conducting *in vitro* studies. Toward this goal BALB/c macrophage cells RAW264.7 were coincubated with increasing concentrations of P85 and 1  $\mu$ g of pDNA for 4 hours at 37°C, 5% CO<sub>2</sub>, washed with PBS and media replaced. After 16 hours cells were washed and lysed with Promega lysis buffer (1X) and supernatant used for quantification of luciferase protein. The assay was performed as follows: 10  $\mu$ l of the supernatant was added to luminometric tubes and supplemented with 100  $\mu$ l of luciferase substrate solution (Promega,

Madison, WI). Light emission was measured with a luminometer (Promega, Madison, WI) for a period of 20 s. Surprisingly, we found that 1% P85 when mixed with 1  $\mu$ g plasmid DNA increased transgene levels upto 2000 times compared to DNA alone in RAW 264.7 macrophages *in vitro* (**Figure 43**). DNA alone and GenePORTER3000 transfection reagent were used as negative and positive controls respectively.

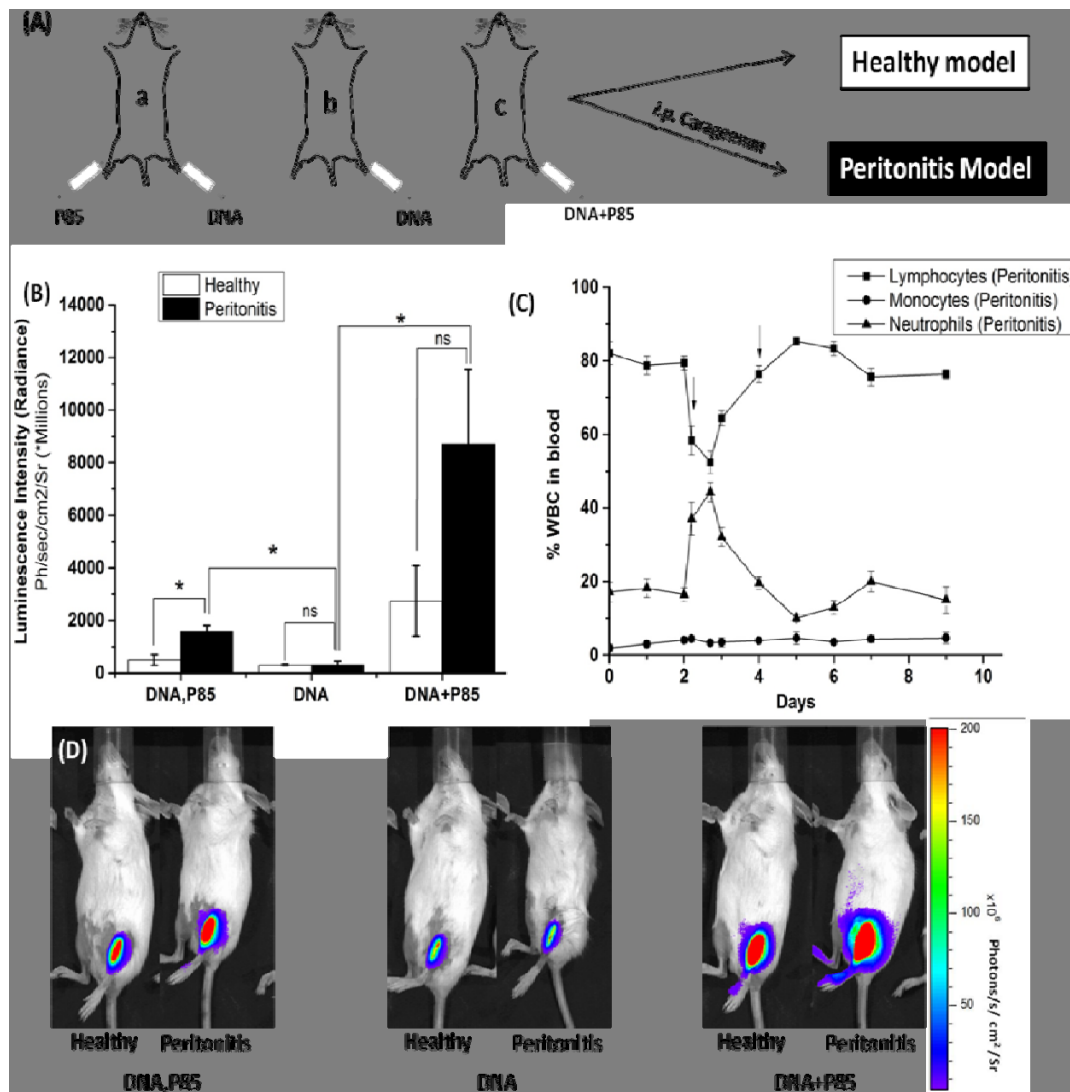


**Figure 43. Quantitative data of *in vitro* luciferase activity:** RAW264.7 macrophage cells were coincubated with 1 $\mu$ g gWIZluc and different concentrations of Pluronic P85. Cells were exposed to P85+DNA for 4 hours in serum free media and assay was performed after 16 hour of incubation. Data are mean $\pm$ SEM (n=3) p values are obtained by means of student's t test.

We studied another model of acute distal inflammation (i.e. peritonitis) to explain the generality of increase in gene expression because of inflammation. In peritonitis distal inflammation model, *i.p* injection of filter-sterilized  $\lambda$ -Carrageenan (1mg/200 $\mu$ l PBS) was performed on 0 day and 2<sup>nd</sup> day followed by *i.m.* injections of plasmid DNA in healthy leg (t.a.) a day after generation of inflammation. The level of inflammation in peritonitis was studied by quantifying percent WBC's in blood by vetscan hematology analysis of serial blood samples (**Figure 44C**) from tail vein and compared to blood samples from healthy animals. DNA injections were performed with/without Pluronic P85 (0.3% w/v) and compared to healthy animals. In order to separate the effect of Pluronic on gene expression, Pluronic and DNA were injected separately in left and right hind limb, respectively. Luciferase reporter protein expression was monitored by injecting D-luciferin (150 mg luciferin/kg body weight) *i.p.* followed by non invasive *in vivo* bioluminescent imaging using Xenogen IVIS 200 imaging system.

Similar to the previous results, in DNA+P85 group, an increased gene expression was observed in the presence of distal inflammation (in this case peritonitis) compared to healthy animals. Again this increase was limited to DNA/Pluronic group and was not observed in animals injected with naked DNA (**Figure 44B** black bars and white bars of DNA group). Interestingly, Pluronic when separated by space (DNA and P85 injected in separate legs) significantly increased gene expression both in healthy and peritonitis animals compared to DNA alone (**Figure 44B and D**). Infiltration of immune cells (e.g. neutrophils, macrophages etc) have long been correlated with repair and regeneration of muscle tissue. Increase in gene expression in the presence of P85 could be explained by increased immune cell infiltration and their role in increasing the transgene expression and tissue repair. Therefore, we further attempted to understand the role of

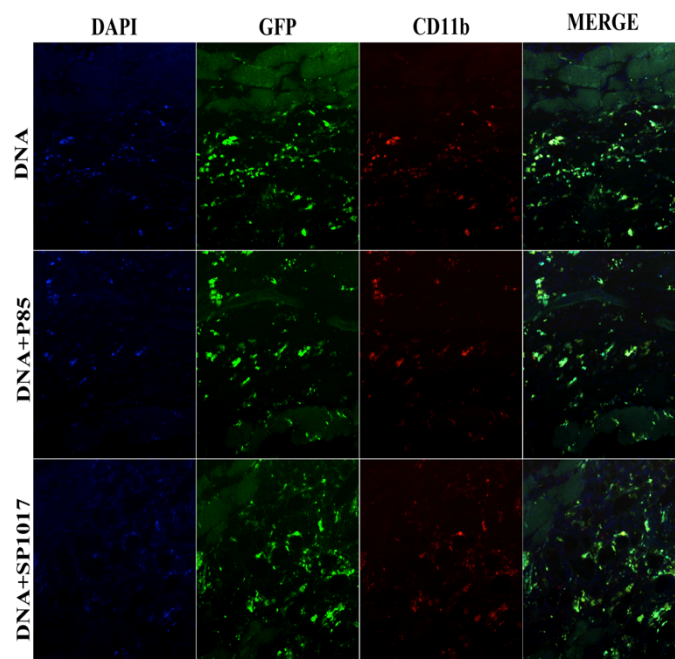
macrophage in gene transfer in *in vivo* (mice hindlimb ischemia model, MHLIM) and *in vitro* models (co-culture of macrophage with myoblast and myotubes).



**Figure 44.** Scheme of experiment (A) Involves 50ul injection of P85 (0.3% wt/vol) and DNA alone (10  $\mu$ g) in separate muscles (a), DNA alone (b), and DNA+Pluronic P85 (c) in *tibialis anterior* muscle of healthy and peritonitis inflammation model. *In vivo* gene expression after 4 days of *i.m.* injections (quantified by bioluminescence imaging) (B): DNA injections were performed in *tibialis anterior* muscle of healthy mice (white bars) and peritonitis model (black bars). Kinetics of WBC count (C) in peritonitis model (solid lines) and healthy mice (dotted lines B). 30-50ul peripheral blood was collected at different time points by tail clip method and percent white blood cells was determined by VetScan HM5 hematology system. Data are mean  $\pm$ SEM (n=5). Representative IVIS images (D). Data are mean  $\pm$ SEM (n=3). ns is non significant at p value of 0.05 \* p < 0.05

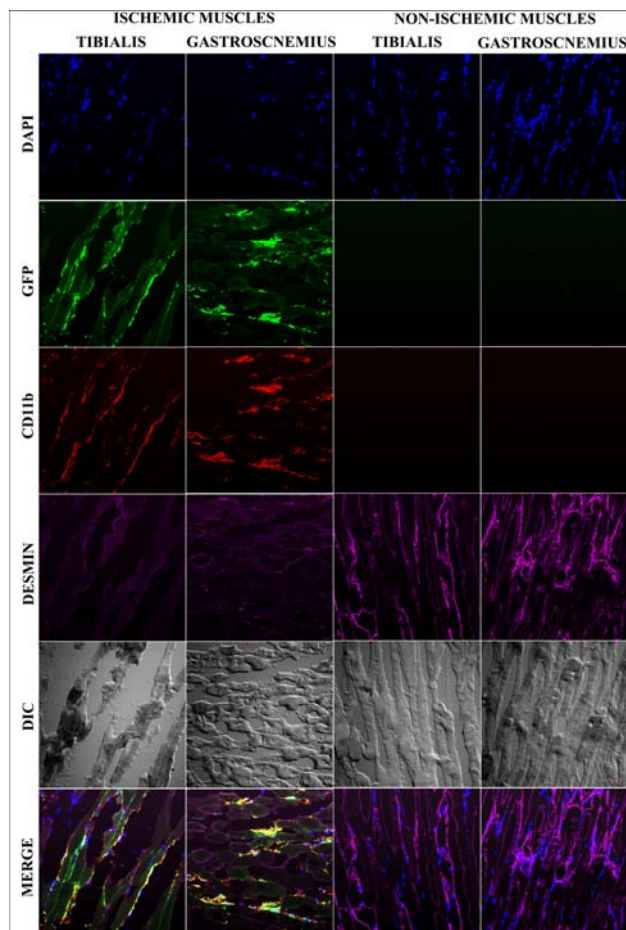
Colocalization of GFP expression and cell specific markers was studied to determine what cells are expressing the transgene after intramuscular injection of DNA. Plasmid DNA encoding GFP with/without Pluronics was injected into the ischemic *t.a.* muscle of MHLIM. The muscle specimens were harvested 4th day after injection and individually processed for immunohistochemistry. The results of this experiment suggest that in the presence of P85/SP1017 the plasmid DNA is also taken-up by antigen presenting cells (**Figure 45**) in addition to muscle cells.

We further studied the possibility of horizontal transfer of nucleic acids. (DNA/mRNA/protein) from APC's to adjacent muscle cells upon *i.m.* Pluronic mediated gene delivery. To test this hypothesis, macrophages transfected *ex vivo* with GFP encoding plasmid were adoptively transferred to ischemic mice 48 hours post ischemic surgery by *intrajugular vein (i.j.v)* injection. Ischemic and non-ischemic muscles (i.e. *tibialis anterior* and *gastrocnemius*) were isolated 3 days post DNA injection and processed for immunohistochemistry. 10  $\mu$ m sections were studied for co-localization of GFP expression with macrophages (CD11b) and muscle cells (desmin) using Zeiss LSM 710 confocal laser scanning microscope. As expected, GFP expressing macrophages were recruited to ischemic muscles and not non-ischemic muscles and GFP expression colocalized with CD11b+ve macrophages in ischemic tissues (**Figure 46**). Interestingly, GFP expression was also observed in muscle fibers and it co-localized with muscle cell marker (Desmin). This would imply that the APCs exchange and transfer the expressed protein/nucleic acids across the muscle membranes and thus collectively represents a reservoirs and conduits for the gene expression. Notably there were lower levels of muscle specific marker (desmin) in ischemic muscle compared to non-ischemic muscle which is a characteristic feature of ischemia that results in depletion of desmin marker and was reported in earlier studies too (9, 10). In an important way, macrophages were localized on the close proximities/edges of muscle fibres characterized by intense colocalization of GFP and CD11b marker. This cell-to-cell contact may thus help assist exchange of repackaged nucleic acids or proteins from macrophages across the muscle membranes of otherwise hard to transfect muscle fibers.



**Figure 45.** Confocal imaging of GFP expression in ischemic muscle 4 days after single injection of DNA alone, DNA+P85 (0.6% w/v) or DNA+SP1017 (0.1% w/v). The color staining corresponds to nucleus (blue); macrophage/myeloid cells (Anti Cd11b) (red); and GFP (green). The GFP expression was assayed by immunohistochemistry using polyclonal rabbit anti-GFP primary antibody. The last panels in each row present digitally superimposed images of three preceding panels to visualize the colocalization (yellow or white). The images were taken with Zeiss 710 confocal laser scanning microscope using 20x objective.





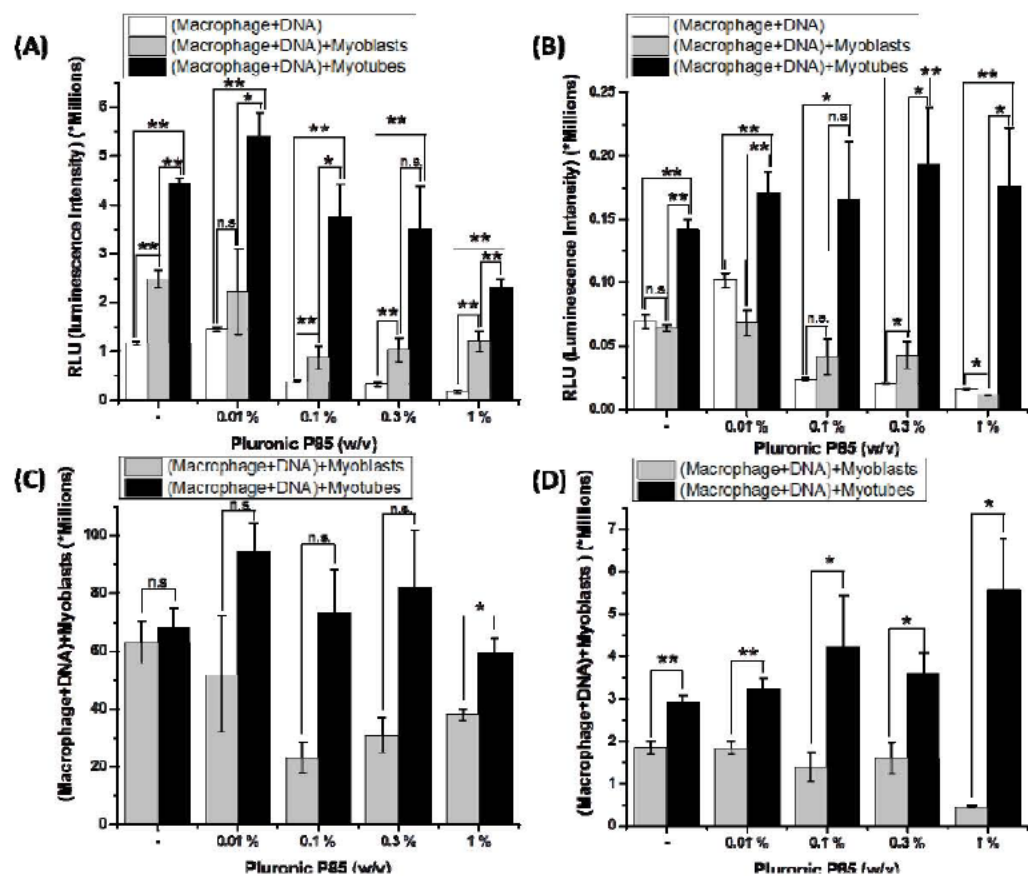
**Figure 46.** Confocal imaging of ischemic and non ischemic muscles of ischemic mice: RAW 264.7 macrophages were transfected ex vivo and injected in BALB/c mice 48 post ischemia surgery by *i.v* injection. Tissues were isolated 3 days post injection and 10um sections of frozen tissues were processed for immunohistochemistry. The color staining corresponds to nucleus (blue), GFP expression (green), CD11b cell marker (red), desmin muscle marker (cyan), phase contrast image (DIC). The last panels in each row present digitally superimposed images of five preceding panels to visualize the colocalization (yellow or white). The images are representative of 3 sections per muscle and 3 mice per group. The images were taken with Zeiss 710 confocal laser scanning microscope using 20x objective. Scale bar represents 50um.

We developed a co-culture model to demonstrate the importance of cell-to-cell contacts whereby *in vitro* transfected macrophages (Mac) were co-cultured on monolayer of post-mitotic differentiated myotubes (MT) and precursor myoblasts (MB). Our aim was to understand whether transfer of nucleic acids can occur from transfected macrophages to host cells (myoblasts or myotubes). We utilized two different plasmids with different promoters: gWIZ-luc plasmid with a constitutive promoter CMV for macrophage transfection and pDesmin-lucia plasmid with cell specific (myoblast and myotube) promoter desmin. When the nucleic acids are transferred, the gene expression should increase upon co-culture, if not there will be no increase in expression. Moreover, if the nucleic acids are transferred and promoter is constitutive, reporter gene will be expressed in both macrophages and co-culture monolayer (MB /MT) so the fold increase in reporter protein expression would be smaller than if the promoter is myoblast/myotube specific (Desmin promoter). In the latter case only MT or MB cells will express the genes (promoter specific expression) and not macrophages, so the fold increase would be more pronounced compared to former. Moreover, because desmin expression increases upon differentiation of myoblasts to myotubes, an increase in gene expression would be even higher in Mac+MT compared to Mac+MB co-culture. Comparison of these two systems served as an internal control to determine the increase in gene expression and affirm the mechanism of gene transfer.

Macrophages were transfected with commercial lipoplex kit genePORTER3000 and membrane bound lipoplexes were removed by rinsing transfected macrophages twice with 1mg/ml heparin sulphate. Macrophages were resuspended in complete media or CM (DMEM supplemented with 10% FBS) and 100,000 cells/well were cultured on top of 50,000/well C2C12 myoblasts or C2C12 myoblasts derived myotubes (day 8 post differentiation). After 2 hours when macrophages adhere to myoblast/myotube monolayer, the co-culture was rinsed thrice with serum-free media to remove traces of serum. The co-culture was then exposed to increasing concentrations of Pluronic P85 (0.01, 0.3, 1.0% w/v) for 2 hours.

In CMV plasmid co-culture model total gene expression increased 2 times in Mac+MT compared to Mac+MB and this fold increase remained the same with increasing concentrations of Pluronic P85 (both total and normalized gene expression) (**Figure 47A** and **C**). Total gene expression was always higher in co-culture compared to Mac alone when CMV plasmid was used (**Figure 47A**). On the other hand, in desmin plasmid co-culture model total increase in gene expression was 2 times in Mac+MT compared to Mac+MB, and these folds increase in gene expression was significantly higher with increasing concentration of Pluronic P85 (16- and 13-times higher for total and normalized gene expression, respectively). As expected, the gene expression levels in macrophage alone were always similar to Mac+MB and, hence, confirm the desmin promoter dependent expression in Mac+MT (**Figure 47B**). Increase in gene expression in Mac+MT co-culture can only be explained by desmin specific expression and happened only when nucleic acids were transferred horizontally from Mac to MT.

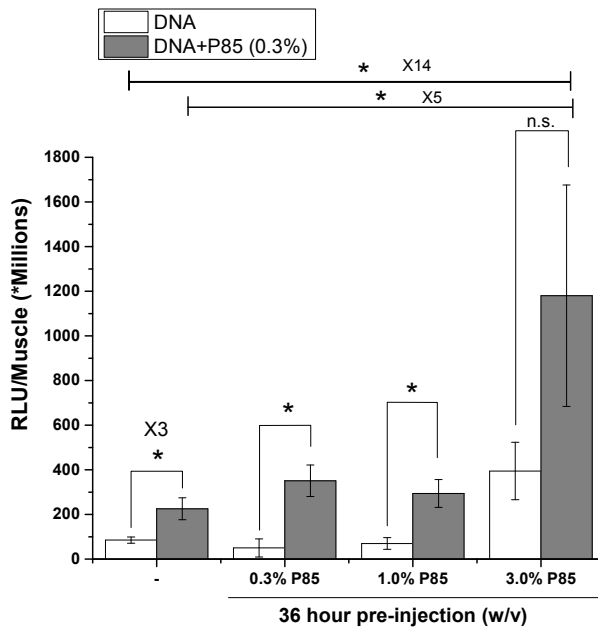
Although, total gene expression was used to compare the levels of gene expression in co-culture (Mac+MB/MT) compared to Mac alone (**Figure 47A** and **B**), little fold increase in CMV and significant high fold increase in desmin specific gene expression was also observed in normalized data (**Figure 47C** and **D**), which further increased in P85 concentration-dependent manner. We also observed lower levels of normalized reporter protein upon co-culture compared to macrophage alone. This observation can be explained by the Fas-L Fas-R mediated apoptosis induced upon co-culture of macrophages on myoblast/myotube which is an evolutionary mechanism to reduce the inflammatory macrophages after the inflammation is resolved in muscle tissue.



Pluronic P85	Fold increase (total expression)		Fold increase (total expression/total protein)	
	(Macrophage+DNA)+Myoblast v/s (Macrophage+DNA)+Myotube	(Macrophage+DNA)+Myoblast v/s (Macrophage+DNA)+Myotube	(Macrophage+DNA)+Myoblast v/s (Macrophage+DNA)+Myotube	(Macrophage+DNA)+Myoblast v/s (Macrophage+DNA)+Myotube
	CMV promoter	Desmin promoter	CMV promoter	Desmin promoter
-	2	2	1	2
0.01%	2	3	2	2
0.1%	4	4	3	3
0.3%	3	5	3	2
1.0%	2	16	2	13

## Year 5

We demonstrated re-injection of P85 36 hours before DNA injections and not 5 days, increased the gene expression significantly compared to DNA or DNA+P85 injected group (without pre-injection). The levels of gene expression were measured by *in vivo* IVIS imaging from 1-50 days (kinetics of gene expression) and semi quantitative analysis was performed to show the differences in the groups. The aim of this experiment was to repeat the experiment and quantifying expression levels of luciferase in muscle homogenates (instead of IVIS) by luciferase assay (**Figure 48**).



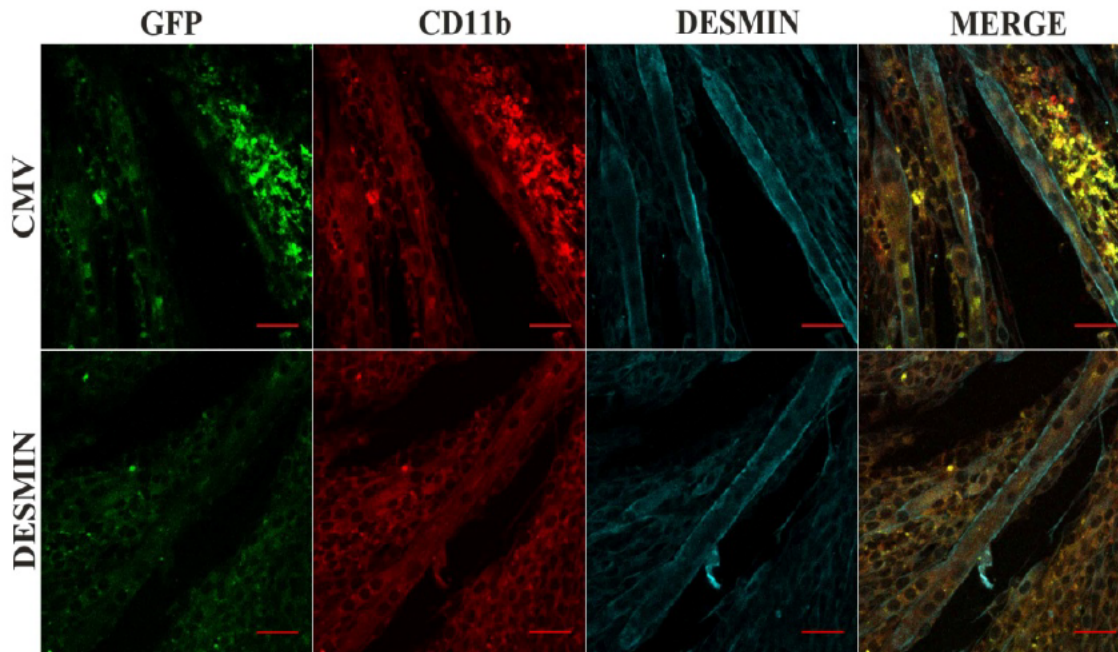
The results demonstrated the trends of increase in gene expression were similar to IVIS imaging results previously reported. **Figure 48** shows about 3-fold increase in gene expression for DNA+P85 (0.3%) (no pre-injection) treatment compared to DNA alone (no pre-injection). After pre-injection with 3% P85, expression levels of DNA+P85 (0.3%) increased 5 times compared to DNA+P85 alone (no pre-injection) and 14 times compared to DNA alone (no pre-injection) (**Figure 48**). The data also shows that gene expression of naked DNA alone after 3% pre-injection likewise increased but the observed change was not statistically significant compared to naked DNA alone (without pre-injection). It should be noted that gene expression increase after pre-injection of P85 and its profile as a function of P85 concentration had a bell shape with a maximum at 3% with lower levels at higher (10%) and lower (0.3 % and 1%) concentrations of Pluronic P85.

Aforementioned scheme of intramuscular injections can be employed to further increase the levels of gene expression, which may result in higher titers of antibody response for DNA vaccination.

We also evaluated the role of macrophages (MPs) in muscle transfection. Our earlier data on co-culture of macrophages with myotubes/myoblasts (MTs) showed increase in total gene expression in both CMV and desmin promoter models. These results led to the hypothesis of muscle cell transfection through macrophages. The role of MPs in muscle transfection was evaluated by co-culturing GFP transfected-macrophages with myotubes for 72 h followed by imaging with Ziess confocal 700 imaging system (**Figure 49**). Specifically, MPs were plated 30



min before transfection and then transfected with GFP-DNA using genePORTER3000 transfection reagent as per manufacturer recommended protocol. After 4 h of transfection cells were rinsed twice with 1mg/ml heparin sulphate followed by PBS rinse. After 1 h 15,000 GFP transfected MPs were plated on MTs (day 7 after differentiation) in 8-chamber well. On day 3 after co-culture, cells were fixed and labeled with the appropriate antibodies and imaged under confocal 700 microscope.



GFP expression in MTs confirmed the delivery of DNA/protein from MPs to MTs upon co-culture for 72h (**Figure 49**). These data may suggest that co-administration of Pluronic block copolymers (e.g. P85) leads to recruitment of MPs to the site of injection (both with and without pre-injection in muscles) that following transfection delivers the DNA to MTs thus resulting in higher transfection efficiencies in Pluronic-assisted gene delivery. These findings can be utilized in platform C to transfect monocytes *in vivo* by targeted BICs (mannose coated), which can then be trafficked to inflammation site resulting in muscle transfection.

## **Platform C**

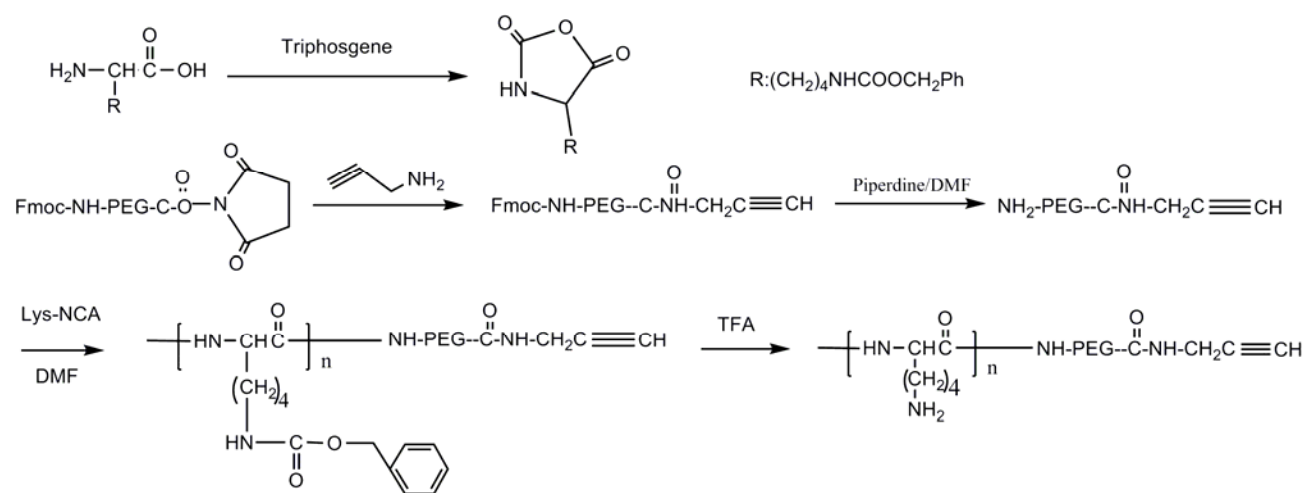
### **Year 1**

According to the milestones the synthesis of the cationic copolymers based on the polypeptides was initiated. Specifically, one sample of cationic graft-copolymer based on polylysine (PLL-g-PEG) has been already synthesized and characterized. The PLL-g-PEG graft copolymer was prepared by reacting succinimidyl derivative of poly(ethylene glycol) (PEG) propionic acid

( $M_w=5000$ , NOF Corporation) with PLL ( $M_n=12,000$ , Sigma) in phosphate buffer for 2 hrs at room temperature. The product was purified by exhaustive dialysis (SpectraPor membrane, molecular weight cutoff size 12-14 kDa) and characterized by  $^1\text{H}$  NMR spectroscopy using  $\text{D}_2\text{O}$  as a solvent on a Varian 500 MHz spectrometer. From  $^1\text{H}$  NMR spectra of the synthesized PLL-g-PEO, the numbers of PEO chains grafted onto a PLL backbone was calculated to be 1.55. Reverse titration was carried out to determine the concentration of amino group in PLL-g- PEO solution, which was determined to be 6 mM.

The synthesis of the cationic block copolymers based on the polypeptides and biodegradable polymers based on polyanhydrides was initiated.

The step-by-step procedure for the synthesis of PEG-*block*-PLL copolymer containing targeting groups was developed as presented in **Figure 50**.



**Figure 50.** Scheme of synthesis of alkyne-modified PEG-*b*-PLL copolymer.

At first the monomer,  $\omega$ -carbobenzyloxyl-L-Lysine N-carboxylanhydride ( $\omega$ -carbobenzyloxyl-L-lysine- NCA) was synthesized by the treatment of the suspension of  $\omega$ -carbobenzyloxyl-L-lysine in THF at 50°C until the clear solutions were formed. The resulting Lys-NCA was then recrystallized from THF/hexane mixture twice and its structure was confirmed by  $^1\text{H}$ -NMR.

The synthesis of amino-PEG-alkynyl was started from the commercial Fmoc-protected PEG-succinimidyl ester (M.w. 5,000, Creative PEGWork). It was allowed to react under dry conditions with propargyl amine to yield Fmoc-NH-PEG-propargyl which is subsequently was deprotected using piperidine/DMF mixture. The product was then used directly as a macroinitiator in lysine-NCA polymerization performed in DMF, under oxygen-free condition at 40 °C, reaction time was 72 hours followed by deprotection of  $\alpha$ -amino group of lysine using TFA. The structure of the prepared copolymer was confirmed by  $^1\text{H}$ -NMR and GPC analysis. Specifically, one sample of PEG(114)-*b*-PLL(70) has been already synthesized and characterized.

The synthesis of biodegradable copolymers based on hydrophilic  $\alpha,\omega$ -bis(*p*-carboxyphenoxy)triethylene glycol (CPTEG) and hydrophobic 1,6-bis(*p*-

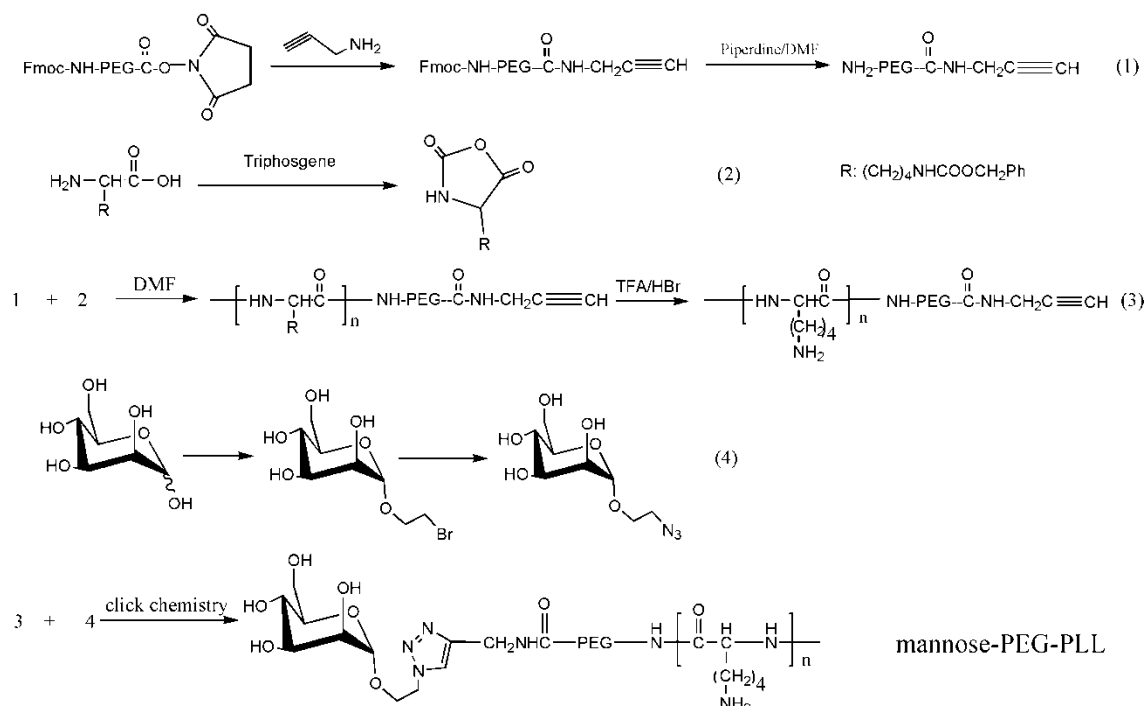
carboxyphenoxy)hexane (CPH) [CPTEG:CPH copolymers] involves the synthesis of CPH and CPTEG monomers. To synthesize the CPTEG monomer, in a typical experiment, 45 mL of triethylene glycol, 100 mL of toluene, 300 mL of dimethyl formamide, and 0.897 mol of potassium carbonate were mixed in a round-bottom flask placed in an oil bath at 170°C. The addition of toluene allowed the azeotropic distillation of water from the reaction mixture prior to the reaction. Next, 0.684 mol of 4-p-fluorobenzonitrile was added and allowed to react overnight at 150°C. After cooling, all the solvents were removed using a rotary evaporator. The resulting dinitrile solution was hydrolyzed with a mixture containing equal volumes (50 mL) of water and acetic and sulfuric acid. The reaction was carried out at 160°C under a nitrogen atmosphere. The resulting diacid was precipitated using 1 L of deionized water. A white powder was obtained after successive washes with acetonitrile.

For polymer synthesis, in a typical experiment, 2 g of the monomer and 100 mL of acetic anhydride were added to a round-bottom flask and reacted for 30 min at 125°C. The acetic anhydride was removed in the rotary evaporator, and the resulting viscous liquid was polymerized in an oil bath at 140°C, under vacuum (0.03 torr) for 90 min. The polymer was isolated by precipitating from methylene chloride into petroleum ether in a 1:10 ratio. The copolymer compositions synthesized were 20:80, 50:50, and 80:20 CPTEG:CPH. Additionally, homopolymers of CPH and CPTEG were synthesized.

The purity of the monomers and polymers was verified using <sup>1</sup>H NMR spectra obtained from a Varian VXR-300 MHz NMR spectrometer and infrared spectra obtained from a Nicolet 6700 FT-IR spectrometer. Number-average molecular weights were estimated by end group analysis from <sup>1</sup>H NMR spectra. Perkin Elmer DSC 7 and DMA were used for the thermal characterization. The samples were heated in two cycles from 20 to 110°C at a rate of 5°C/min in the DSC. The NMR and IR studies confirmed the structure of the polymers. The molecular weights of the polymers synthesized ranged from 4000 to 14,000 g/mol. All the CPTEG-containing polymers have glass transition temperatures below room temperature, making them rubbery polymers.

The synthesis a series of cationic block copolymers based on polylysine containing targeting groups were synthesized and characterized according to the milestones.

The following synthetic scheme was implemented for the synthesis of PEG-*block*-PLL copolymers containing targeting groups:



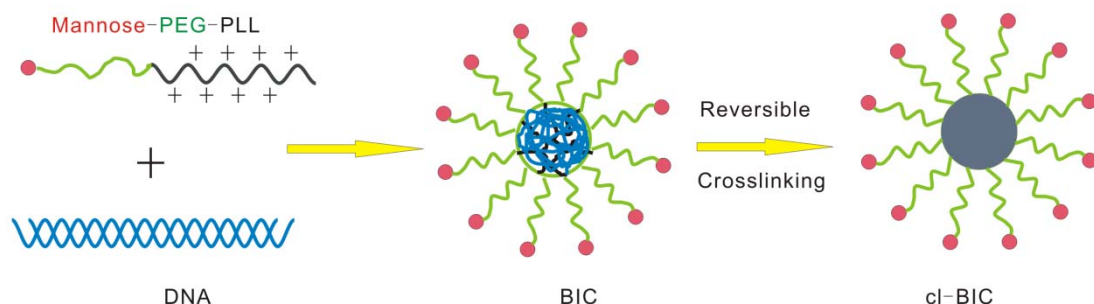
**Figure 51.** Step-by-step procedure for the synthesis of alkyne-modified PEG-*b*-PLL copolymer

A commercial Fmoc-protected PEG- succinimidyl ester (M.w. 5,000, Creative PEGWork) was used to synthesize a propargyl-modified intermediate following the deprotection of terminal amino group. The resulting amino-terminated PEG was used as macroinitiator for the polymerization of  $\omega$ -carbobenzyloxyl-L-lysine- NCA. The polymerization was performed in DMF, under oxygen-free condition at 40 °C, reaction time was 72 hours followed by deprotection of  $\omega$ -amino group of lysine using TFA .The length of PLL block was controlled by varying the monomer/macroinitiator feed ratio. The structure of the prepared copolymers was confirmed by <sup>1</sup>H-NMR and GPC analysis. Specifically, the following samples of PEG(114)-*b*-PLL(70) has been already synthesized and characterized.

Composition	PDI	Mannose %
Mannose-PEG <sub>114</sub> -PLL <sub>62</sub>	1.27	87%
Mannose-PEG <sub>114</sub> -PLL <sub>150</sub>	1.25	72%
Mannose-PEG <sub>114</sub> -PLL <sub>206</sub>	1.19	95%

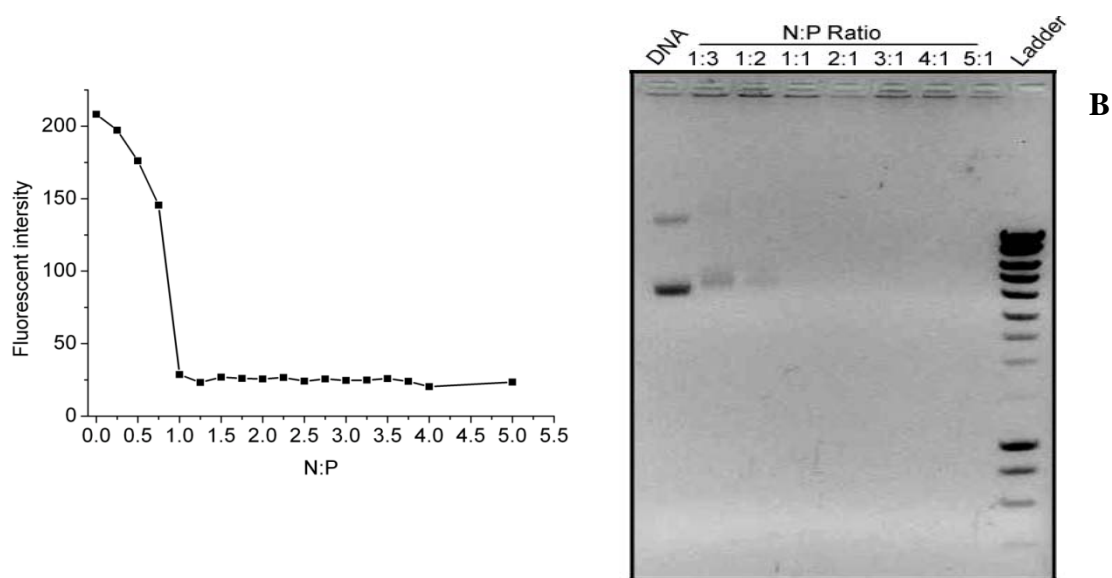
## Year 2

Next, we characterized a series of targeted BIC/DNA compositions. BICs were prepared using mannose-capped poly(ethylene glycol)-block-poly(L-lysine) diblock copolymer mannose-PEG<sub>114</sub>-PLL<sub>62</sub> (the numbers in subscripts show the relative number of monomer units in the blocks) and gWIZ-LUC plasmid DNA (Luc-DNA) via our previously identified method (**Figure 52**).



**Figure 52.** Schematic representation of the synthesis of crosslinked (cl) -BICs

To verify the formation of BICs and condensation DNA by mannose-PEG<sub>114</sub>-PLL<sub>62</sub>, ethidium bromide (EtBr) exclusion assay was used. Briefly, DNA was mixed with ethidium bromide (DNA: EBr= 5:1 w/w) prior to particle formation. Then cationic block polymer mannose-PEG<sub>114</sub>-PLL<sub>62</sub> were added to the mixture at predetermined N: P ratio (the molar ratio of amine groups of PLL to DNA phosphate groups), then vortexed for 20 seconds immediately, and incubated for 30min at R.T. Upon mixing this systems remained transparent and no precipitation was observed over entire range of N:P ratios examined. The resulting solutions were added to two wells on a white opaque 96 well plate and fluorescence was measured with 360 nm excitation and 590 nm emission. As shown in **Figure 51A**, addition of cationic mannose-PEG<sub>114</sub>-PLL<sub>62</sub> copolymer to DNA-EB complex resulted in quenching of the EB fluorescence due to the displacement of EB by the copolymer. Fluorescent intensity decreased gradually with the increase of N:P ratio, and leveled off when the N:P ratio exceeded 1:1. These data suggest that maximal quenching of EB fluorescence was observed at equivalency of the polycation (PLL) and DNA charges (N:P = 1).



**Figure 53A.** Binding of mannose-PEG<sub>114</sub>-PLL<sub>62</sub> with plasmid DNA examined by ethidium bromide exclusion assay. Data are presented as mean fluorescence intensity of the samples. **B.** Agarose gel retardation assay. The final concentration of DNA was 15µg/ml



Formation of BIC between DNA and mannose-PEG-PLL was also investigated by retardation assay using agarose gel electrophoresis (**Figure 53B**). Migration of the DNA in the gel was retarded as the amount of the copolymer was increased. This demonstrated that mannose-PEG-PLL copolymer was binding to DNA, neutralizing its charge. At stoichiometric composition (N:P = 1) complete neutralization of DNA by the copolymer was achieved and no DNA bands were observed at N:P ratio larger than 1, consistent with the results from EB exclusion assay (**Figure 53A**). Thus, both methods confirmed that cationic block copolymer mannose-PEG-PLL can form complexes with DNA.

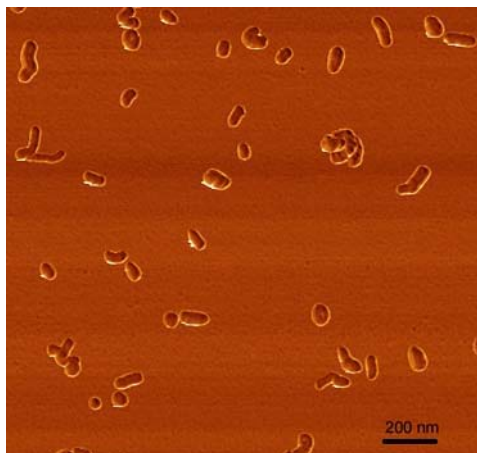
BICs were further cross-linked using dithio-bis-(succinimidyl propionate), DSP, a reducible crosslinker containing disulfide bonds. Complexes were prepared in the presence of the excess of block copolymer at N:P ratio equal 3. Predetermined amounts of DSP dissolved in DMSO were added to the formed BIC to induce the cross-linking. The amount of DSP was calculated on the basis of the targeted cross-linking ratio defined as the total amount of reactive groups in DSP solution versus total number of Lys residues in mannose-PEG-PLL copolymer. It is important to note that the extent of targeted cross-linking represents the maximum theoretical amount of cross-linking that can take place, rather than the precise extent of cross-linking, which is expected to be lower. Mixtures were incubated for 1 h at room temperature followed by dialysis against 10mM HEPES buffer (pH 7.4) to remove excess amount of cationic polymers. Hydrodynamic diameter and zeta-potential of BICs were determined by dynamic light scattering (Malvern Zetasizer, Malvern Instruments Ltd., Malvern, UK). All measurements were performed in automatic mode, at 25°C. Software provided by the manufacturer was used to calculate the size, polydispersity indices and zeta-potential of BICs (**Table 7**). All measurements were performed at least in triplicate. All examined BICs formed small particles with averaged diameters in the range of 90-100 nm with relatively narrow size distribution (polydispersity indices were below 0.2) and nearly neutral surface charge (**Table 7**).

**Table 7.** Physico-chemical characteristics of mannose-PEG<sub>114</sub>-PLL<sub>62</sub>/Luc-DNA BICs (N:P 3:1)

Degree of crosslinking (cl) with or without dialysis	Particle Size (nm)	Polydispersity Index	Zeta Potential (mV)
0%	99.0 ± 1.4	0.198±0.008	1.70±0.65
25% non-dialyzed	114.3±1.4	0.208±0.010	0.09±0.32
50% non-dialyzed	101.4±0.5	0.163±0.010	-6.23±0.07
75% non-dialyzed	103.8±0.4	0.204±0.010	-7.58±0.51
25% with dialysis	83.6±0.6	0.243±0.022	2.13±0.84
50% with dialysis	169.8±4.7	0.290±0.032	-8.26±0.33
75% with dialysis	103.6±0.7	0.147±0.007	-6.72±0.31

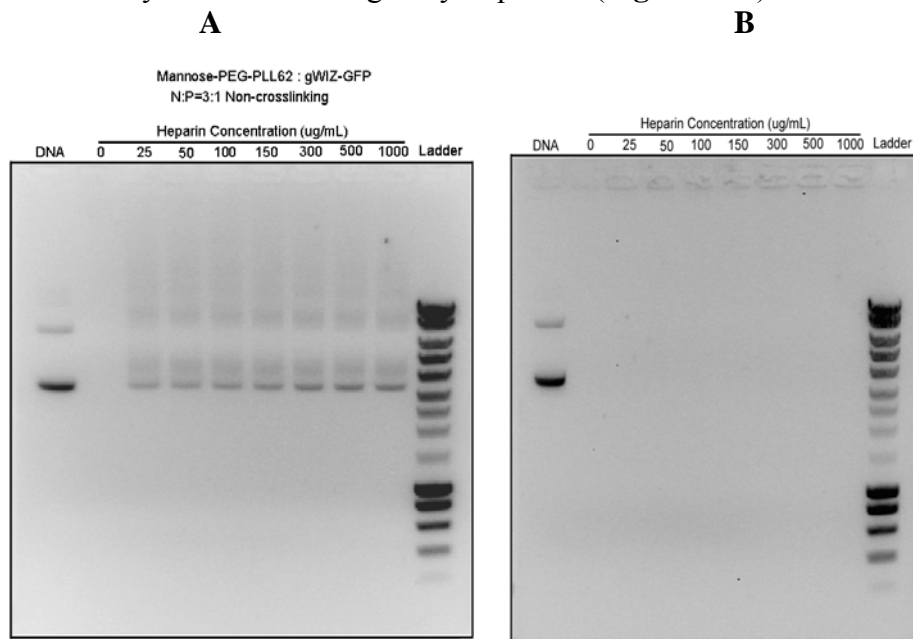
The morphology of cl-BICs was further examined by atomic force microscopy (AFM) using a Multimode NanoScope IV system operated in a tapping mode. For sample preparation, BICs were deposited on mica surface for 2 min, followed by surface drying under argon atmosphere. The AFM images were processed and the widths and heights of the particles were determined by using Femtoscan software (Advanced Technologies Center, Moscow, Russia). A representative

AFM image of cl-BIC with 25% targeted degree of cross-linking is shown in **Figure 54** and indicated the formation of slightly elongated complex particles.



**Figure 54.** AFM image of mannose-PEG<sub>114</sub>-PLL<sub>62</sub>/Luc-DNA BICs (N:P = 3) with targeted degree of cross-linking of 25%. Bar is equal 200 nm.

Stability of cross-linked BIC was studied by agarose gel electrophoresis after co-incubation with heparin for 1 hour. Heparin, an anionic polysaccharide with high charge density, can strongly interact with the mannose-PEG-PLL cationic copolymer and may substitute DNA from non-crosslinked BICs which could subsequently be visualized by EtBr staining by gel electrophoresis (**Figure 55A**). As expected, DNA was substituted from the non cross-linked BICs by heparin. On the contrary, the formation of cross-links between the polycation chains in BIC rendered the DNA release from the cross-linked BICs even in the presence of high concentrations of heparin suggesting that the stability of the BIC was greatly improved (**Figure 55B**).

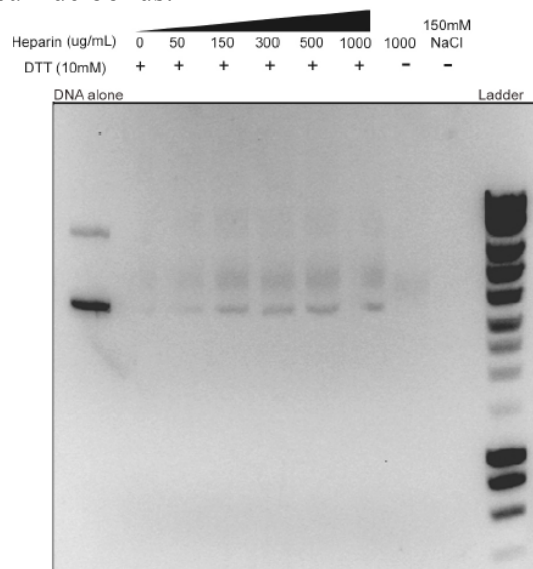


**Figure 55.** Stability of non-crosslinked BICs (**A**) and crosslinked clBICs (**B**) (N:P =3:1). Both types of BICs were incubated with heparin for 1 hour prior to agarose gel electrophoresis; the values represent the concentration of heparin (µg/ml).

The introduced cross-links in clBIC contained reversible disulfide bonds that are relatively stable in extracellular environment but degrade in reducing environment inside the cells. Redox



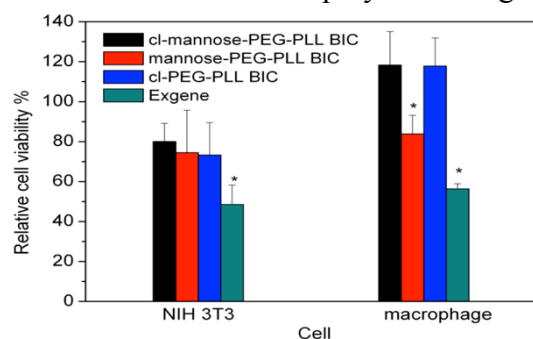
sensitivity of cross-linked BIC (complexes were prepared using DNA plasmid encoding GFP protein) and was confirmed by incubating complexes with dithiothreitol, DTT, for 30 min at 37°C followed by challenging them with heparin and analyzing by gel electrophoresis (Figure 56). The bands corresponding to DNA were observed in case of complexes treated with DTT confirming reduction of disulfide bonds.



**Figure 56.** Stability of cross-linked BICs in the presence of 10 mM DTT (C). BIC samples (mannose-PEG<sub>114</sub>-PLL<sub>62</sub>/GFP-DNA ) were incubated with heparin for 1 hour prior to agarose gel electrophoresis. The values represent the concentration of heparin (µg/ml). Complexes were prepared at N:P = 3 with 25% targeted degree of cross-linking and purified by dialysis.

The cl-BICs were also incubated with NaCl solution for 30min, and ran on the agarose gel. According to **Figure 56** (second lane from the right), no DNA was substituted by heparin, suggesting that cross-linked BICs were stable at physiologically relevant concentrations of salt (NaCl, 150mM).

Possible cytotoxicity of mannose-PEG-PLL/DNA complexes in both NIH/3T3 cells and mouse bone marrow derived macrophage (BMM) cells was evaluate using standard MTT assay (Figure 57). For macrophages, both cl-mannose-PEG-PLL BICs (black bars) and cl-PEG-PLL BICs (blue bars) exhibited much lower cytotoxicity as compared to non-crosslinked mannose-PEG-PLL BICs (red bars). This might ascribe to the removal of excess of cationic polymers from cl-BICs by dialysis. Furthermore, we observed significantly lower toxicity of our BICs than that of commercial transfection polymeric reagent Exgene 500 (green bars).



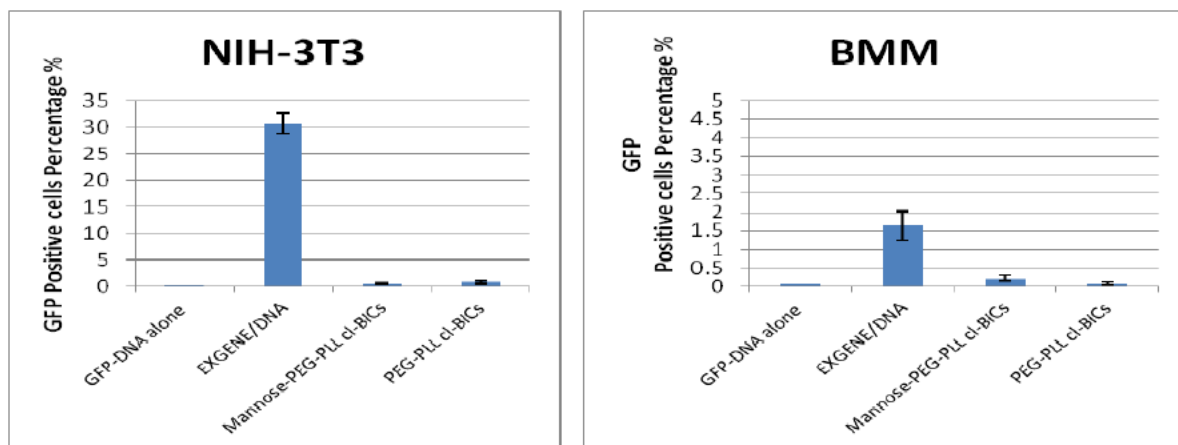
**Figure 57.** Cytotoxicity of BICs to NIH/3T3 cells and BMMs.

For further in vitro transfection experiments, BMMs cells were used as the model cell line corresponding to high mannose receptor expression while NIH/3T3 fibroblasts with low level of mannose receptor expression were used as a negative control. The physicochemical characteristics of BICs used for transfection experiments are presented in **Table 8**.

**Table 8.** Physicochemical characteristics of various BICs used in transfection experiments

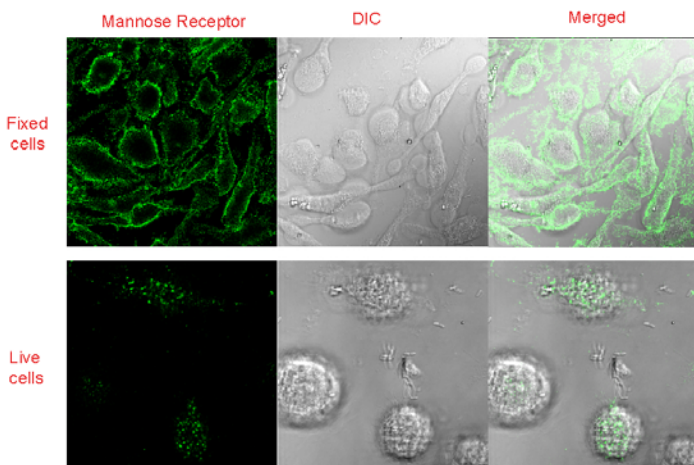
Polymers in Luc-DNA complex	Particle Size (nm)	PDI
EXGENE 500 (PEI)	336.0±18.4	0.344±0.028
Mannose-PEG <sub>114</sub> -PLL <sub>62</sub>	91.7±2.4	0.156±0.010
PEG <sub>114</sub> -PLL <sub>50</sub>	87.3±0.4	0.173±0.028
Mannose-PEG <sub>114</sub> -PLL <sub>62</sub> (cl)	106.1±3.9	0.168±0.009
PEG <sub>114</sub> -PLL <sub>50</sub> (cl)	87.9±1.6	0.148±0.013
Mannose-PEG <sub>114</sub> -PLL <sub>62</sub> (cl and dialysis)	95.5±2.4	0.202±0.011
PEG <sub>114</sub> -PLL <sub>50</sub> (cl and dialysis)	92.2±1.8	0.213±0.015

As it seen from **Figure 58**, transfection efficiency of BIC was low compared to Exgene 500/DNA complexes and no difference was observed between targeted and nontargeted BICs in both cell models. However, mouse BMM cells did not express considerable level of mannose receptors as was determined by FACS analysis via staining mannose receptors with specific antibody (only 2.3% of BMM cells were positive for mannose receptor). Therefore, selecting appropriate macrophage cells that express considerable amount of mannose receptors is important for testing the targeted gene delivery strategy.

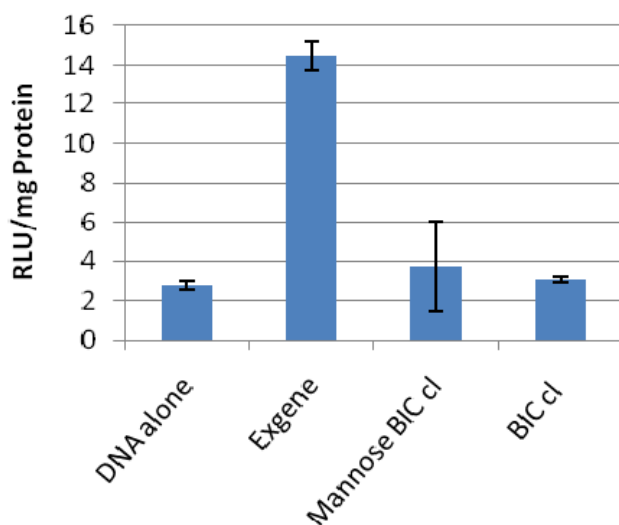


**Figure 58.** Transfection of NIH-3T3 cells and BMMs with various BICs.

An interesting option is human monocyte-derived macrophage cells. These macrophage cells were differentiated from human monocytes in the culture medium supplemented with macrophage colony-stimulating factor (MCSF). Primary mouse monoclonal mannose receptor antibody was applied at 1:20 dilution and goat-anti-mouse IgG conjugated with Alexa 488 dye (green color) were used as secondary antibody. A strong staining of mannose receptors was observed on the surface of these human macrophage cells (**Figure 59**). However, there was no significant difference of luciferase expression between the cells transfected with mannose functionalized BICs and non-targeted BICs (**Figure 60**).



**Figure 59.** Confocal images of human monocyte-derived macrophage cells stained for mannose receptors (green).



**Figure 60.** Transfection of human monocyte-derived macrophages with Luc-DNA plasmid incorporated into cross-linked mannose-decorated BIC or into BIC without mannose targeting moieties.

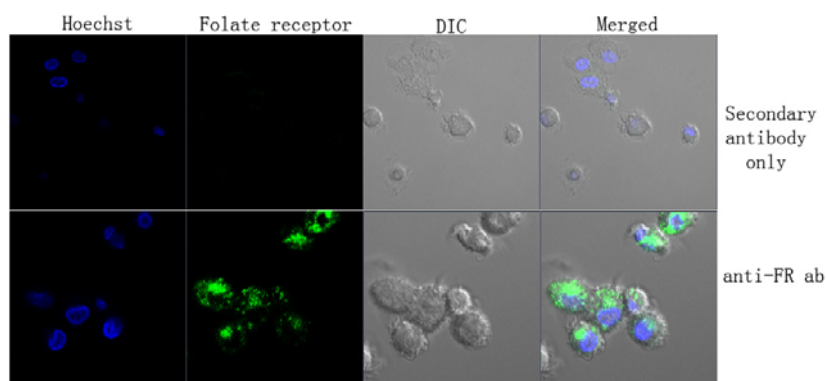
BICs formed by cationic copolymers with different lengths of PLL block and various density of surface mannose targeting moieties were tested.

### **Year 3**

As mannosylated BICs presented no significant advantages over non-functionalized BICs as either the mouse bone marrow macrophages (BMM) expressed moderate level of mannose receptors or the transfection efficiency in human monocyte-derived macrophages was low (**Figure 58** and **59**). Multiple recent publications have demonstrated the feasibility of folate mediated active targeting delivery of genes to macrophages (8, 11-14), therefore, we applied Folate functionalized BICs as an alternative strategy.

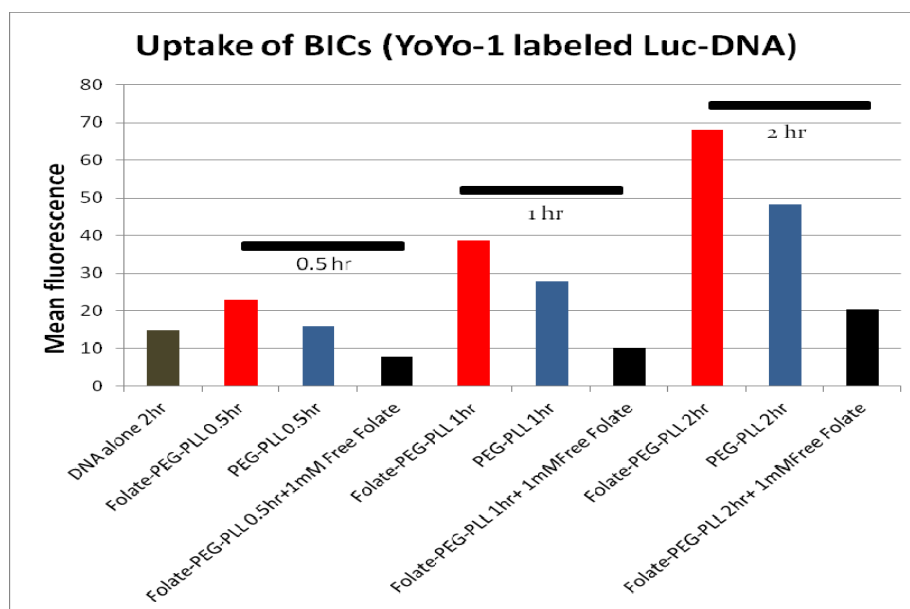
#### **Alternative strategy 1: Folate functionalized BICs (Folate-BICs)**

**Folate receptor levels in macrophage cells.** To confirm the expression of folate receptors, we immunostained folate receptors in human monocyte-derived macrophage cells. As shown in **Figure 61**, folate receptor (green staining) is highly expressed in these human macrophage cells.



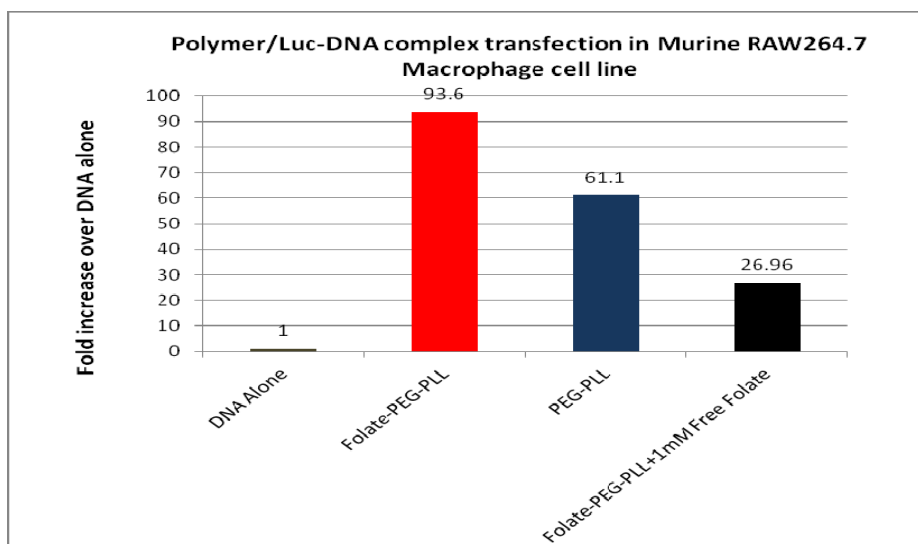
**Figure 61.** Folate receptor staining in human macrophage cells. Blue, nucleus; Green, folate receptor; Grey, DIC. Primary antibody: anti-FR rabbit polyclonal antibody; secondary antibody: Goat-anti-rabbit alexa 488 conjugated.

**Folate-BICs.** Folate-BICs were produced by mixing Folate functionalized cationic copolymers Folate-Poly(ethylene glycol)-b-Poly(L-lysine) (Folate-PEG-PLL) with opposite charged DNAs, the same approach to make mannose-BICs. BICs using PEG-PLL copolymer without folate targeting moiety (non-Folate-BICs) were used as a control. Cellular uptake of these BICs was determined by labeling DNA with YoYo-1. A clear trend (**Figure 62**) followed 0.5, 1 and 2 hours incubation times showing higher uptakes of Folate-BICs (Red bar) as compared to non-Folate-BICs (Blue bar), while concomitant addition of 1mM free folate was sufficient to compete out the uptakes of Folate-BICs (Black bar).



**Figure 62.** Uptake studies of YoYo-1 Labeled DNA in BIC complexes.

When transfecting RAW264.7 murine macrophage cells (**Figure 63**), Folate-BICs (Red bar) exhibited nearly 96 fold increase of luciferase expression over DNA alone (Brown bar), and approximately 1.5 times higher than the non-Folate BICs (50% increase) (Blue bar). In addition, co-incubate with free folate (Black bar) abolished the DNA transfection efficiency.

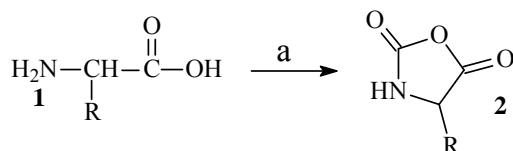


**Figure 63.** Transfection efficiency of different BICs in Murine Macrophage RAW264.7 cells.

Above results with merely 50% increase in cellular uptakes and gene expression for Folate-BICs over non-Folate-BICs prompted us to re-verify the polymer quality. Significant amount of impurities in the previously synthesized polymer products were identified possibly ascribing to degradation or imprecise purification, which demands the synthesis of a new batch high purity polymers. Proposed new synthetic pathway is described as following.

**Methods and Schematics of the synthesis of Folate-Poly(Ethylene glycol)-Poly(L-Lysine) polymers (Folate-PEG-PLL<sub>50</sub>) [15]**

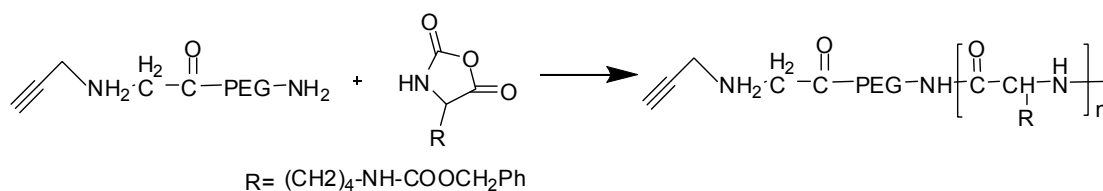
Synthesis of monomer (Lys-NCA) (Scheme 1): 5.0g (0.0178 mole) Nε-Carbobenzyloxy-L-Lysine (Lys(z)) was dissolved in 60 ml anhydrous THF and stirred to form suspension at room temperature (RT). The nitrogen was bubbled through the mixture during synthesis (about 3h). About 2.0g (0.00674mole) of triphosgene was dissolved in 15 ml of anhydrous THF and added to the above suspension drop by drops as injection to reaction solution. The mixture was heated up to 50°C and left to proceed at reflux conditions. After the mixture become transparent, the solution was precipitate in anhydrous n-hexane (about 225 ml) and then stored at -20°C overnight. Crude product formed during precipitation was recrystallized twice with anhydrous THF/Hexane mixture. The white solid (Lys-NCA) monomer was dried under vacuum for 24 h and characterized by <sup>1</sup>H-NMR (CDCl<sub>3</sub>).



Scheme 1

Synthesis of Fmoc-PEG-Propargyl and NH<sub>2</sub>-PEG-Propargyl: The 0.55g (100μmol, 1eq) Fmoc-PEG-NHS were dissolved in 3mL extra dry dichloromethane(DCM). Dilute 80μL (1200μmol, 12eq) propargylamine to 5mL DCM and mix with 3mL Fmoc-PEG-NHS solution. Mixture was stirred overnight at 4 °C and next day evaporate DCM and dissolve products in 15mL H<sub>2</sub>O followed by dialysis against DD H<sub>2</sub>O overnight with molecular weight cutoff 2000. 447.5mg white Fmoc-PEG-Propargyl products were obtained via lyophilization. Deprotection of Fmoc group was realized by 20% piperidine/DMF and for 2 h at RT, then the solvent were removed by rotary evaporation. Crude products were dissolved in DCM, precipitated with diethyl ether, and filtrated/dried under vacuum.

Synthesis of Propargyl-PEG-PLL(Z) (polymerization): The polymerization of propargyl-PEG-PLL (Poly-L-Lysine) were completed (Scheme 2, n=50). Briefly, 0.2g of NH<sub>2</sub>-PEG-Propargyl was dissolved in 5ml of anhydrous DMF and 3μl of triethylamine (TEA) was added to this solution upon stirring to convert NH<sub>3</sub><sup>+</sup> to NH<sub>2</sub>. Mixture was stirred continuously with nitrogen bubbling through the solution and temperature was maintained at about 35-40°C. Calculated amount of Lys-NCA (612mg) which is equivalent to 50 lysine units per PEG chain was dissolved in 2ml of anhydrous DMF and injected dropwise to the reaction mixture. The reaction ampule was then argon bubbled, sealed and kept with stirring for 2 days. Reaction products were precipitated using excess of diethyl ether. The sample was filtered and the solid precipitate was re-dissolved in 5 ml DMF and precipitated with excess amount of ether. The precipitation procedure was repeated 3 times and solid polymer products were dried upon vacuum for 24 h followed by 1H-NMR analysis. Removal of Z protecting group was realized in trifluoroacetic acid (TFA) using a 33% hydrobromic acid solution in galatic acetic acid. The reaction mixture was drop-wise added to excess of cold diethyl ether to precipitate deprotected polymers. The precipitates were redissolved in methanol and precipited with diethyl ether twice.



Scheme 2

Synthesis of 2-Azidoethylfolate: 443.42 mg folic acid (FA, 1 mmol) was dissolved in 20 mL anhydrous DMSO. 250.40 mg N,N'-dicyclohexylcarbodiimide(DCC, 1.2 mmol) and 13.04 mg 4-dimethylaminopyridine(DMAP, 0.1 mmol) were dissolved in 3 mL anhydrous DMSO and added to the FA solution under stirring at 40-41°C for 15 minutes. The activated FA was added dropwise to a stirring solution of 347.5 mg 2-Chloroethylamine hydrochloride (3 mmol) in 10 mL anhydrous DMSO. Afterwards 0.2086 mL TEA(1.5 mmol) was added and the reaction mixture was stirred over night at room temperature in the dark. The urea byproduct was removed by filtration through glass wool and the approx. 30 mL solution was diluted with 30 mL water and 1 mL 1N NaOH, and precipitated with 1 mL 6 M HCl and dried under reduced pressure(0.140 mbar) over night. The solid was redissolved in 10 mL anhydrous DMSO and 195.20 mg NaN<sub>3</sub>(3 mmol) were added and stirred for 3 h at 49-53 °C. The product was precipitated in a large excess of diethyl ether (1:20) and wash 3 times with approximately 20 mL ether and dried yellow solid was obtained.



### Alternative strategy: Liposome/DNA complex (Lipoplex) formulations

A commercial *in vitro* transfection reagent GENEPORERS demonstrated steady high efficiency for DNA transfection to murine macrophage cell line RAW264.7. However, this *in vitro* reagent may not be suitable for *in vivo* translational application and is associated with high toxicity. During the synthesis of Folate-PEG-PLL polymers, we produced various alternative formulations (Lipoplex) with surface modification of Folate-lipids in order to achieve: 1). high transfection efficiency 2). Low toxicity 3). Targeted gene delivery to macrophages 4). *In vivo* injectable formulation 5). simple manufacturing procedures.

Two different cationic liposomes composed of [N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and dioleoylphosphatidylethanolamine (DOPE) (DC-Chol/DOPE molar ratio 1:2) or Dimethyldioctadecylammonium bromide (DDAB) and cholesterol (DDAB/Chol, molar ratio 1:1) were made using the dry film method (14). Briefly, all lipids were dissolved in ethanol. Surface modification were realized by adding either Folate-PEG-DSPE (targeting moiety, 2% molar percentage, PEG molecular weight 5kDa) or PEG-DSPE (non-targeting control, 2% molar percentage) in the mixture, and then the mixture were dried with N<sub>2</sub> gas. The dried thin films were rehydrated with DD water overnight, sonicated for 10 min and then passaged through 0.2µm syringe filter for 10 times. Effective diameter and zeta potential of liposomes were measured (Table 9).

**Table 9.** The effective diameter and zeta potential of cationic liposomes

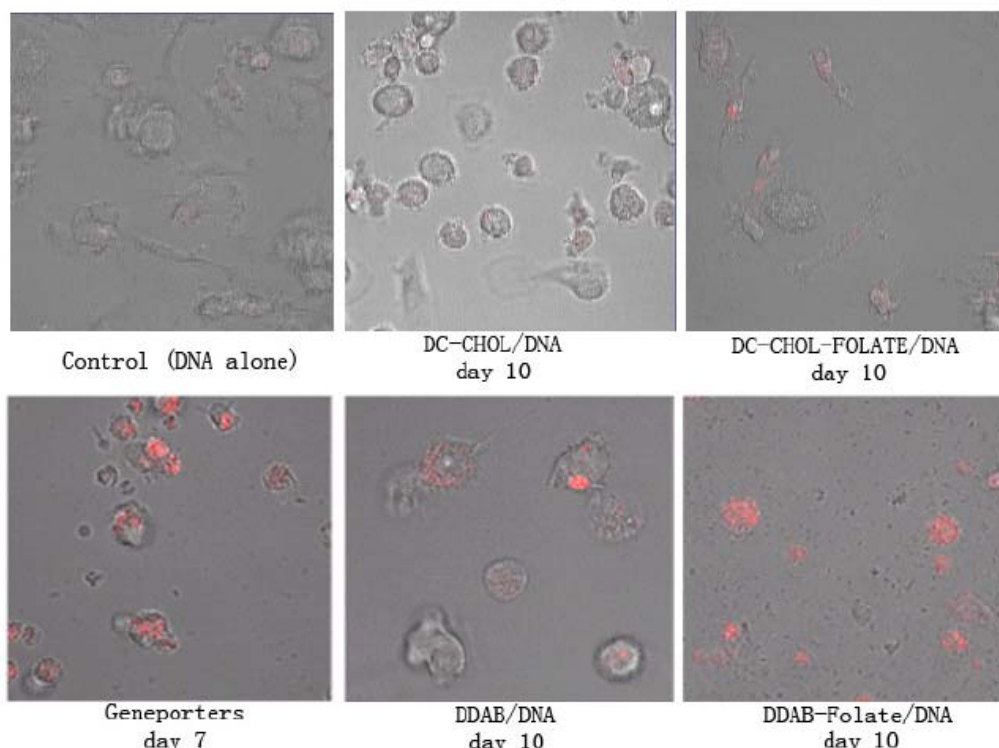
Formulations	Particle Size (nm)	Polydispersity Index	Zeta Potential (mV)
DC-Chol/DOPE(1:2) with PEG <sub>5k</sub> -DSPE	46.2 ± 0.6	0.246±0.006	45.3±0.5
DDAB/Chol(1:2) with PEG <sub>5k</sub> -DSPE	88.3±0.9	0.296±0.023	37.7±0.5
DC-Chol/DOPE(1:2) with Folate-PEG <sub>5k</sub> -DSPE	202.4±32.1	0.426±0.066	29.9±0.2
DDAB/Chol(1:2) with Folate-PEG <sub>5k</sub> -DSPE	239.8±27.1	0.454±0.048	36.5±1.3

The folate modified liposomes showed relative larger effective diameter and higher polydispersity which may require the optimization of folate-PEG-DSPE concentration. Lipoplex can be produced by gentle mixing calculated amount of DNA with cationic liposomes and keep at RT for 20 minutes. Uptakes and transfection efficiency can be determined by adding lipoplex into macrophage cells.

Cationic liposomes composed of Dimethyldioctadecylammonium bromide (DDAB) and cholesterol (molar ratio 1:1) as well as DDAB-Folate/Cholesterol were made. Their complexation with DNA and transfection efficiency were preliminarily determined by adding lipoplex (liposome/DNA complex; charge ratio 6:1) into human monocytes-derived macrophage cells (human blood monocytes treated with macrophage colony stimulation factor (MCSF) for 7days). These macrophage cells were grown in folate free RPMI1640 cell culture medium before adding lipoplex. Tomato red DNA was used to form lipoplexes and the expression of tomato red fluorescence protein was measured under confocal microscope everyday for up to 12 days. As shown in **Figure 64**, control (DNA alone) had no expression of



tomato red while DC-Chol/DNA and DC-Chol-Folate/DNA formulations showed very low level of expression. On the contrary, positive control Geneporters efficiently transfected DNA to macrophage cells with peaking expression time at day 7. It is promising that DDAB/DNA and DDAB-Folate/DNA formulations exhibited comparable transfection efficiency to positive control but only postponed expression peak at day 10.



**Figure 64.** Tomato red protein expressions in human monocyte-derived macrophage cells

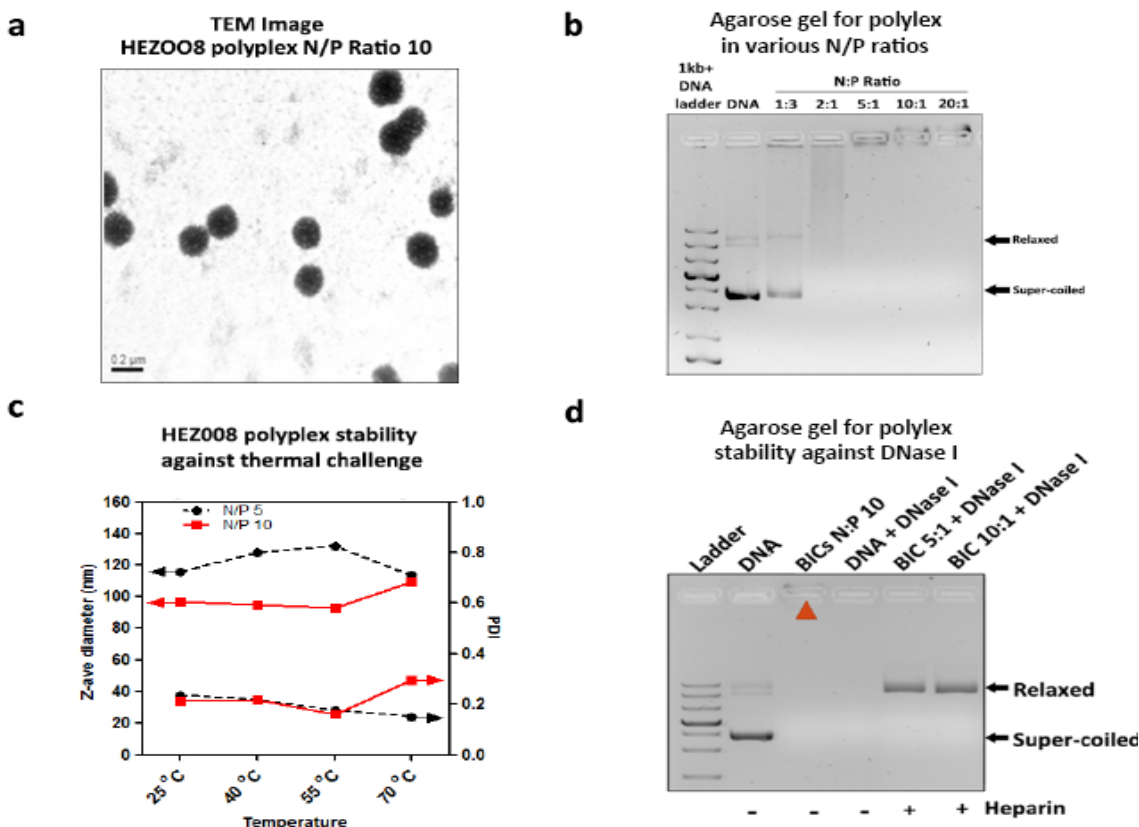
#### **Year 4**

#### **Formation, morphology and stability of Cationic Poly(2-Oxazolines) HEZ008/pDNA polyplexes**

Here we demonstrated spherical morphology and estimate diameter around 100nm of polyplexes exemplified by the Transmission Electron Microscopy (TEM) image of HEZ008/pDNA (N/P ratio 10) (**Figure 63a**). HEZ008 non-targeted polymer was able to condense DNAs and form polyplexes with pDNA, thus retarding pDNA migration on the agarose gel if N/P ratios above 2 (**Figure 65b**).

Stability of HEZ008/pDNA polyplexes is critical factors regarding the translation of this gene delivery platform into *in vivo* application. We subsequently performed a number of stability studies as following. First, we measured temperature stability of HEZ008/pDNA polyplexes at N/P ratio of 5 and 10 by plotting the Z-average diameter and PDI as a function of increasing temperatures from 25°C to 70°C (**Figure 65c**). Interestingly, the Z-ave diameter and PDI of polyplexes were found to be stable throughout the entire analyzed temperature both for 5:1 and 10:1 N:P ratios. We then investigated the stability of polyplexes against DNase I degradation. As shown in **Figure 65d**, pDNAs without complexing was degraded by DNase I while polyplexes at

N:P ratio 5 or 10 preserved pDNAs from enzymatic degradation. When adding heparin, protected pDNAs were released from the complexes and then visualized on the agarose gel under UV light.

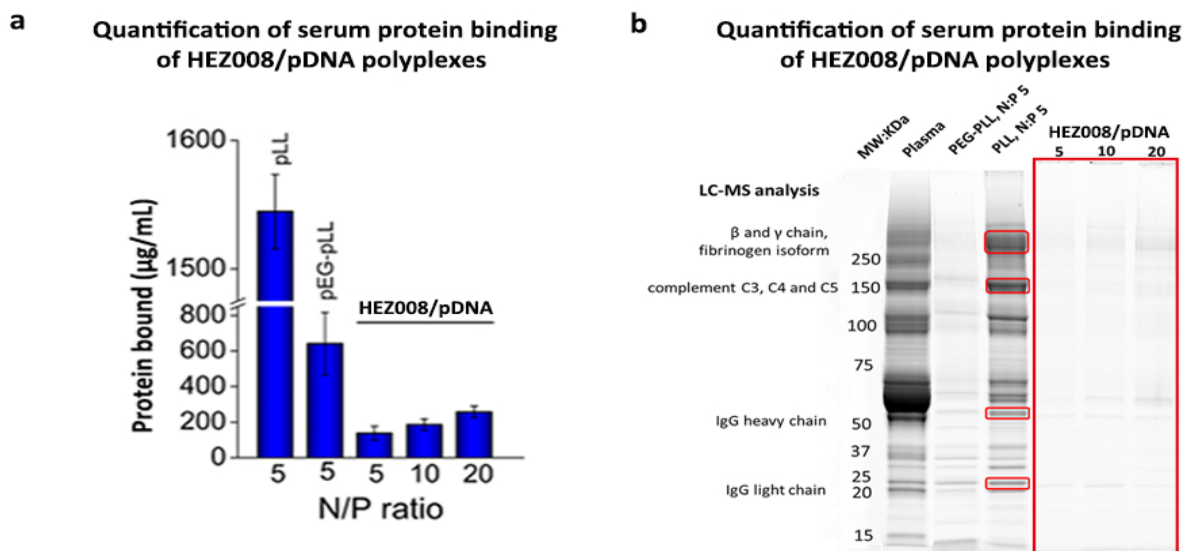


**Figure 65.** Formation, morphology and stability of HEZ008/pDNA polyplexes. (a) TEM image of polyplex at N:P ratio of 10 as a representative result; (b) agarose gel electrophoresis of polyplexes (N:P ratios ranged from 1:3 to 20:1); (c) The Z-average diameter and corresponding PDI of polyplexes formed at N/P charge ratios 5 and 10 against temperature increase from 25 to 40, 55 and 70°C; (d) agarose gel electrophoresis of polyplexes (N:P ratios of 5 and 10) against DNase I digestion: lanes include DNA ladder, DNA, Polyplex N/P 10 without DNase I and Heparin, DNA plus DNase I, polyplex N/P 5 with DNase I plus DNA released by heparin, and polyplex N/P 10 with DNase I plus DNA released by heparin.

### Serum protein binding of HEZ008/pDNA polyplexes

Nanoparticles binding to serum proteins are directly linked to their uptake and further clearance by mononuclear phagocyte system (MPS) *in vivo*. The hydrophilic blocks of poly(2-Oxaoline) [i.e. pMeOx and pEtOx homoblocks] have been previously discovered with anti-fouling or so called “stealth” properties due to the unique chemical structure of POx backbones. This prompted us to evaluate our newly synthesized HEZ008 in this regards when forming polyplexes. We utilized SDS-PAGE gel with SYPRO Ruby staining to show serum proteins that bound to various N:P ratios of polyplexes and found that very low protein binding to our polyplexes (all 5, 10, and 20 N:P ratios) as compared to the complexes formed by poly(ethylene glycol)-b-poly(L-Lysine) (PEG-PLL)/pDNA or poly(L-Lysine) (PLL)/pDNAs (**Figure 66b**). We further quantified serum protein binding via microBCA assay and confirmed that very low serum protein binding by HEZ008/pDNA polyplexes in contrast to other control complexes (**Figure**

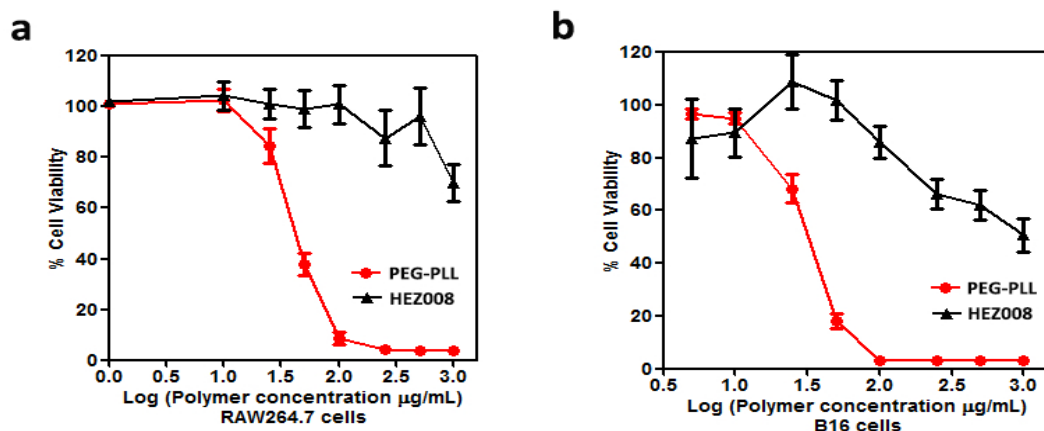
66a), in agreement with the intensity of bound serum protein bands visualized by SYPRO Ruby staining on the SDS-PAGE gel. The major bound proteins were identified from LC-MS analysis as IgG heavy and light chains, complementary C3, C4, and C5, and  $\beta$  and  $\gamma$  chain of fibrinogen isoforms.



**Figure 66.** Serum protein binding of HEZ008/pDNA polyplexes. **(a)** Quantification of bound serum proteins to polyplexes corresponding to **(b)**; **(b)** rat serum protein binding to HEZ/008 polyplexes (large red box) visualized on SDS-PAGE protein gel as compared to PEG-PLL or PLL/pDNA complexes. Small red box indicating major bound proteins identified by LC-MS as  $\beta$  and  $\gamma$  chain of fibrinogen isoforms, complementary C3, C4, and C5, and IgG heavy and light chains.

### Determination of *in vitro* cytotoxicity of HEZ008 Polymers

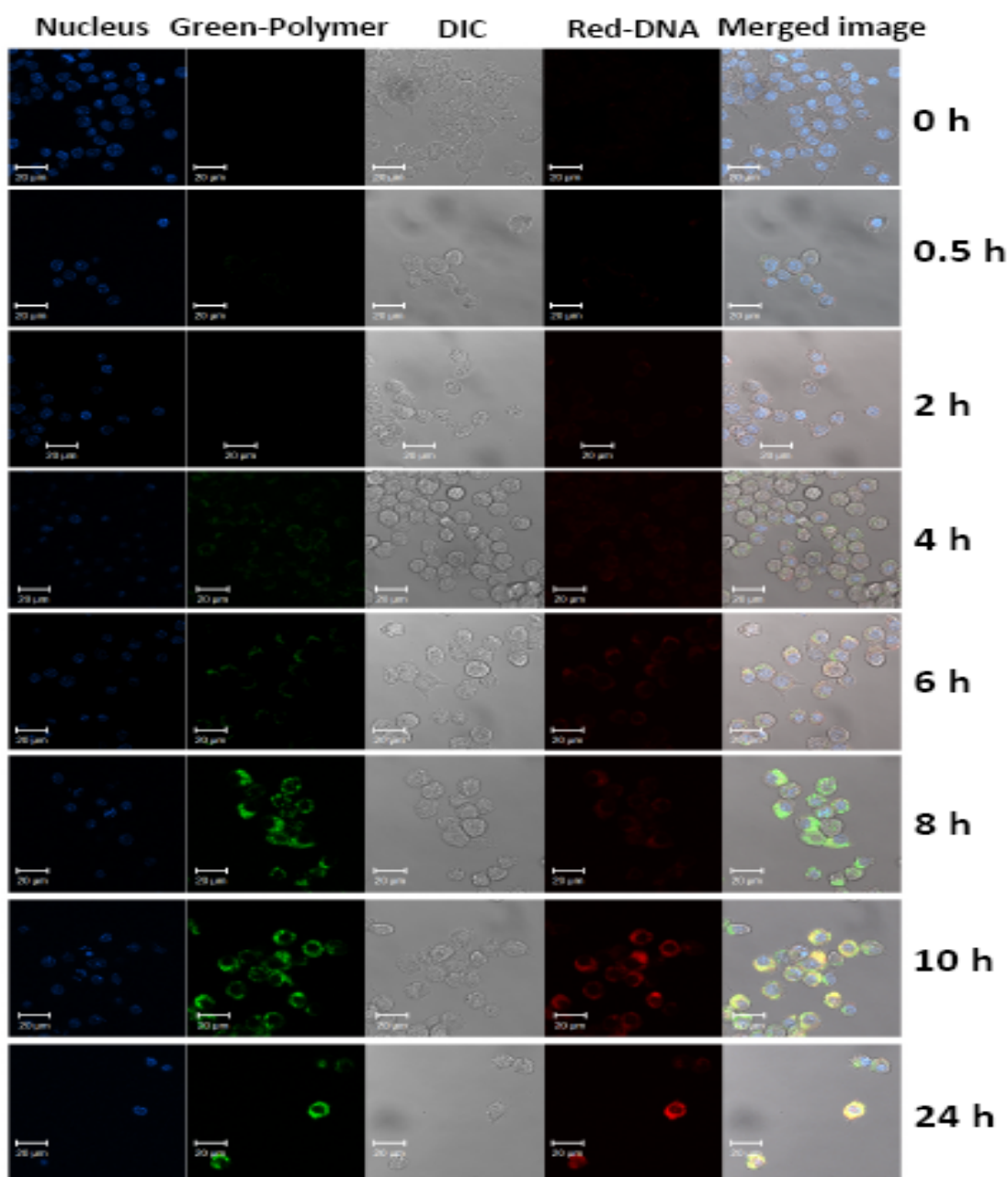
Many cationic polymer based delivery systems were associated with toxicity issues. In order to condense DNA forming stable complexes, large excess of cationic polymers were often used, which may induce cytotoxicity due to their direct interaction with cell membranes. In this respect, we studied *in vitro* cytotoxicities of HEZ008 polymers in RAW264.7 murine macrophage and B16 melanoma cell lines. Cytotoxicities of PEG-PLL were tested in parallel as a comparison. Interestingly, up to 500µg/mL of HEZ008 polymers showed minimum influence on RAW cells viability (**Figure 67a**) while PEG-PLL induced obviate cytotoxicity with an approximate  $IC_{50}$  value equal to 50µg/mL. Similar results were observed in B16 cells (**Figure 67b**).



**Figure 67.** *In vitro* cytotoxicity of HEZ008 polymers in RAW264.7 murine macrophage cells (a) and B16 melanoma cells (b). Cell monolayers were incubated with respective polymer for 24 h before cell viability measurement by MTT assay. Data represents average  $\pm$  S.D. of hexaplicate samples. Red line, PEG-PLL; Black line, HEZ008.

#### Analysis of cellular uptake of HEZ008/pDNA polyplex in macrophage cells

Cellular uptake of HEZ008/pDNA polyplex (pDNA was gWIZ-Luc plasmid DNA, N/P 5) were studied in RAW264.7 macrophage cells using confocal microscopy. For this experiment, the HEZ008 polymer was fluorescence labeled with Carboxyrhodamine. 110 (**Figure 68**, green) using click chemistry while pDNA was visualized using TOTO-3 fluorescence dye (Invitrogen) (**Figure 68**, red). Figure 68 displayed the incubation of polyplex with cells at 0.5, 2, 4, 6, 8, 10 and 24 h time points, respectively. We found that the uptake process in RAW cells was rather slow, and till 4h no significant amount of polyplex were taken up but after 6 h and 8 h they started to enter the cells, and majority of polyplex were taken up after 10 h. Furthermore, polyplex were found very concentrated in the perinuclear region of the cell after 24 h incubation (**Figure 68**); however, significant loss of cell viability was also evidenced for 24 h time point.



**Figure 68.** Cellular uptake of BICs in RAW macrophage cells. Blue, nuclear staining (Hoechst); Green, HEZ008; Red, DNA with TOTO-3 dye.

In this study we demonstrated that the cationic polymer HEZ008 exhibited extremely minimum cytotoxicity and very resistant to serum protein binding when complexed with pDNA. We also found that the HEZ008/pDNA polyplexes were slowly uptaken by RAW macrophage cells (10 h as optimum). The safety and anti-fouling properties encouraged our exploration of HEZ008 as a new gene delivery platform. The HEZ008/pDNA polyplexes showed excellent stability against enzymatic DNase-I digestion as well as thermal challenge. Furthermore, the polyplexes were

very stable upon dilution in buffers and salines as well as storage at 4 degrees for at least 2 weeks as indicated in previous report. The stability of polyplexes we demonstrated here is crucial particularly when applying this technology in biological systems.

The slow uptakes in macrophage cells also offer an opportunity to minimize non-specific uptake *in vivo* but provide a time window to target specific cell populations via attaching affinity ligands on alkyne terminal group via click chemistry tool. The clickable property of the polymer was evidenced by successfully attaching a fluorescence dye and could be further developed extensively for targeting purpose. The stability, uptake and targeting property of mannose-clicked HEZ008 (Man-HEZ008) were explored both *in vitro* and *in vivo* by using mannose receptor overexpressed macrophage cell models.

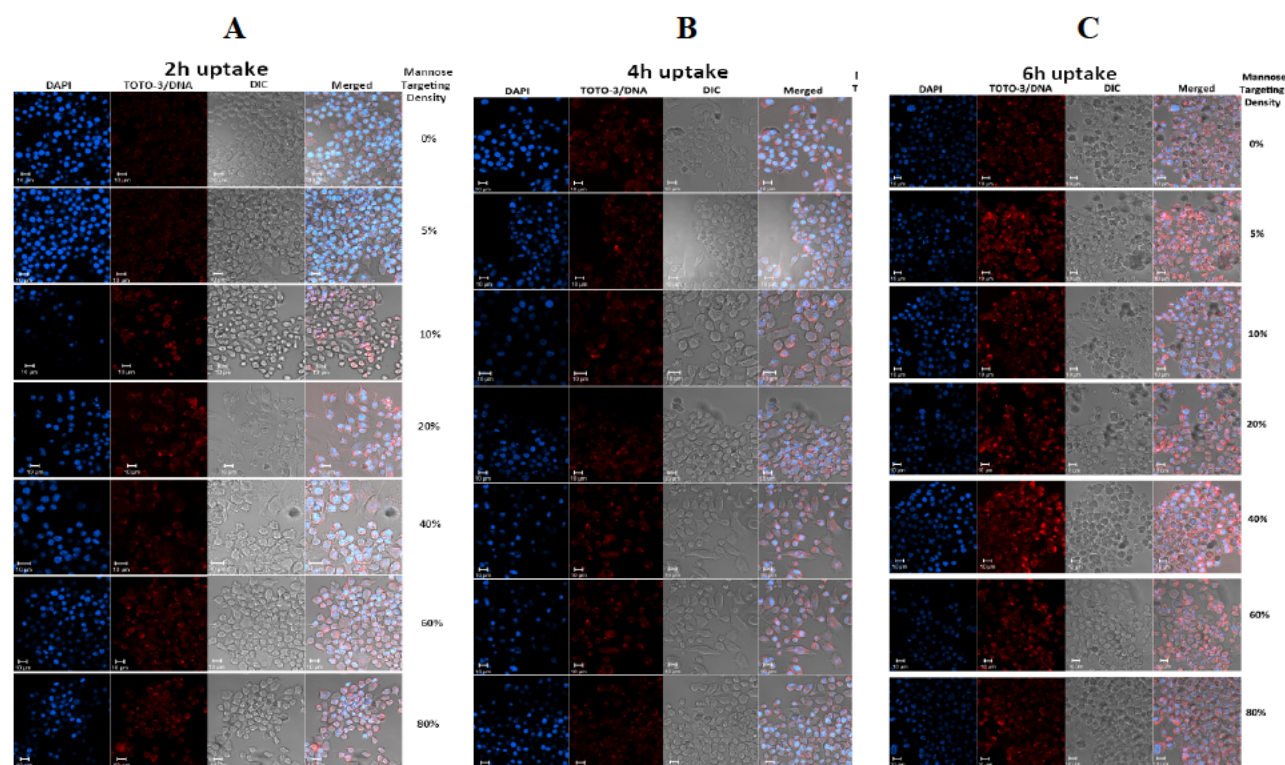
## **Year 5**

### **Uptake of targeted cationic mannosylated-Poly(2-Oxazolines) Man-HEZ008/pDNA polyplexes compared to non-targeted polyplexes HEZ008/pDNA.**

Next, we aimed to identify the optimal density of mannose ligands on the surface of targeted polyplexes. Cellular uptake of Mannose-HEZ008/DNA polyplexes of various density was studied using confocal microscopy while pDNAs (gWIZ-luc DNA) were labeled with TOTO-3 dye (red color in images). Briefly, 8,000/well RAW macrophage cells were seeded in 8-well chamber slide, incubated overnight for cell attachment, and supplemented with MCSF (20ng/mL) for 48hr before adding polyplexes.

Targeted polyplexes with surface mannose density ranging from 0% to 80% (0%, 5%, 10%, 20%, 40%, 60%, and 80%) were produced by complexing pDNA with designated ratios of the mixture of HEZ-008 and Mannose-HEZ008. After 48 hr activation by MCSF, cells were treated with polyplexes for 2, 4 and 6hr and then washed three times with PBS followed by fixation in 4% paraformaldehyde. Fixed cells were visualized under LSM510 confocal microscope. **Figure 69A** indicates that at 2hr, pDNA uptake (red, TOTO-3) was increased when density of mannose in the polyplexes above 10%, peaked at 20% and 40%, and maintained similar level at 60%-80%. From 4hr uptake image (**Figure 69B**), pDNA uptake was increased when density of mannose in the polyplexes above 10%, peaked at 40%, and maintained similar level at 60%-80%. In contrast, pDNA uptake was increased starting from density of mannose in the polyplexes at 5%, 10% and 20%, and peaked at 40%, and uptakes diminished at 60%-80% in the 6hr uptake results (**Figure 69C**).





**Figure 69.** Cellular uptake of targeted HEZ008/pDNA polyplexes in RAW macrophage cells with escalating density of targeting mannose groups from 0, 5, 10, 20, 40, 60 and 80%: (A) 2hr, (B) 4hr and (C) 6hr. Blue, nuclear staining (Hoechst); Red, DNA labelled with TOTO-3 dye; Grey, DIC.

These results provided guidance to the animal studies on DNA vaccines comparing non-targeted polyplexes (HEZ008/pDNA) with targeted polyplexes (Mannose-HEZ008/pDNA) at 40% mannose density.

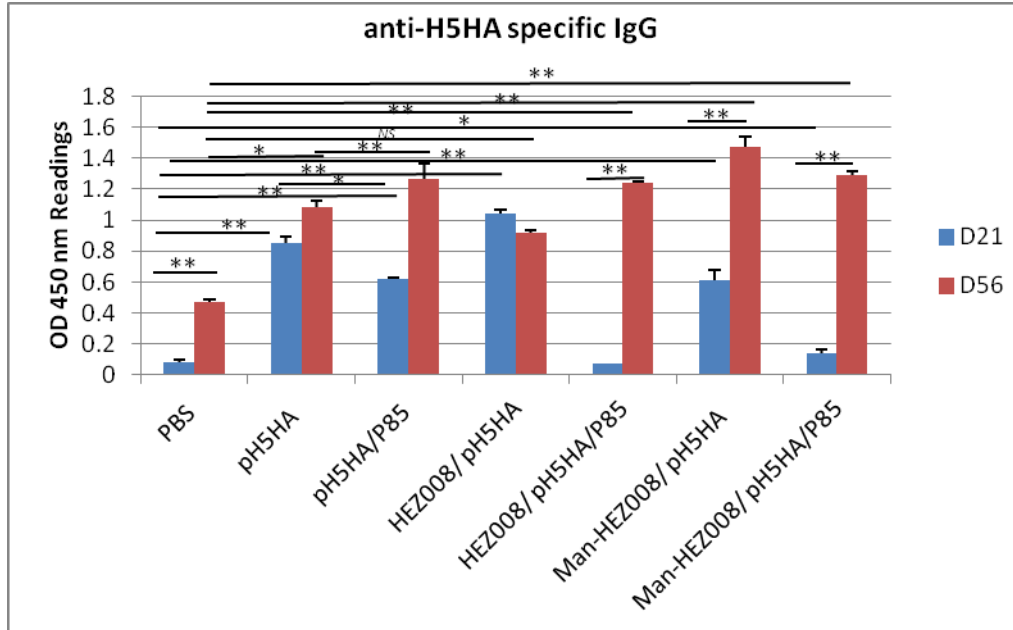
In order to determine whether non-targeted polyplexes (HEZ008/pDNA) and targeted polyplexes (Mannose-HEZ008/pDNA) at 40% mannose density provide any immune responses in animals, groups of BALB/c mice were immunized with these polyplexes. Doses of 50 $\mu$ g of H5HA-encoding pDNA were used for immunization in various formats (naked pDNA or polyplexes).

### H5HA specific antibody responses

To determine the induction of antibody responses against H5HA by polyplexes, ELISA was performed. Non-targeted polyplexes (HEZ008/pH5HA) and non-targeted polyplexes (HEZ008/pDNA) with targeted polyplexes (Mannose-HEZ008/pH5HA) at 40% mannose density formulated with or without 0.3% w/v Pluronic P85 were administered i.m. in tibialis anterior muscles in BALB/c mice (5 mice/group) and boosted on day 28 (D28) with the same formulations. Blood serum samples were collected on day 21 and 56 and performed ELISA (**Figure 70**). The present study demonstrated that pH5HA vaccine formulations with/without Pluronic could induce specific IgG antibodies in mice (**Figure 70**). On the day 21, the IgG antibody level in the naked pH5HA or non-targeted polyplexes and non-targeted polyplexes (HEZ008/pDNA) with targeted polyplexes (Mannose-HEZ008/pH5HA) at 40% mannose density was significantly higher those in the groups received without 0.3% w/v P85 (**Figure 70**). However, on the day 56 the naked pH5HA and polyplexes exhibited significant increase in IgG



antibody compared to PBS or naked pH5HA group with Pluronic P85. The mice vaccinated with HEZ008/pDNA did not show any booster response while all the rest of the groups showed significant booster effect (**Figure 70**). These data indicated that pH5HA DNA vaccine formulations could induce lasting primary antibody responses.



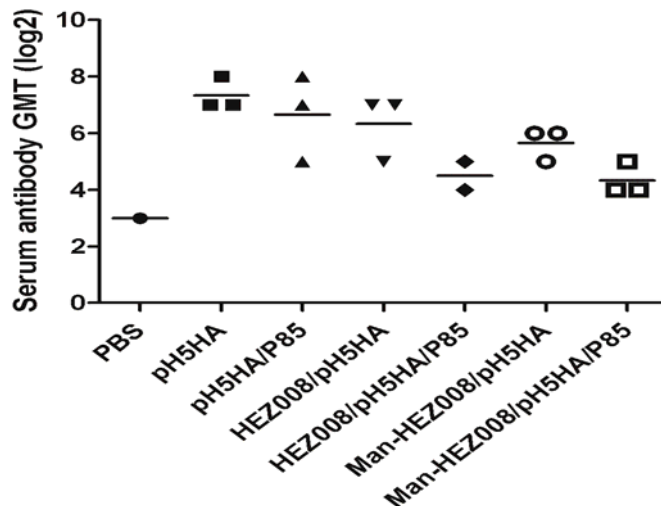
**Figure 70.** Total serum IgG antibody responses to H5HA. 50 µg of pH5HA polyplexed with non-targeted (HEZ008/pH5HA) and non-targeted (HEZ008/pH5HA) with targeted (Mannose-HEZ008/pH5HA) at 40% mannose density and formulated with/without 0.3% w/v Pluronic P85. 50 µL formulations in PBS were administered in tibialis anterior muscles (5 mice/group) and boosted with the same formulations on D28. Sera from individual mice were taken 21 and 56 days after administration of vaccine formulations and were tested for binding to H5 hemagglutinin by ELISA. All data are presented as means ± SEM and analyzed with Student's *t*-test (n=4) at 1:31250 serum dilution. \*\**P*<0.01, \**P*<0.05 and NS is not significant at 0.05 levels.

### Targeted DNA vaccines against H5N1 influenza

In order to determine whether non-targeted polyplexes (HEZ008/pDNA) and targeted polyplexes (Mannose-HEZ008/pDNA) with 40% mannose density provide any immune responses in animals, groups of BALB/c mice were immunized with these polyplexes. Doses of 50 µg of H5HA-encoding pDNA were used for immunization in various formats (naked pDNA or polyplexes). The following vaccination regimen was implemented: animals were vaccinated on day 0 and then boost was given on day 28. Challenge studies were initiated on day 63.

Mice that received 2 doses of pH5HA had higher serum HAI Ab titers against the A/Vietnam/1203/2004 (H5N1) virus compared to PBS injected mice (**Figure 71**). A very similar level of serum HAI Ab titers were observed in the groups of mice those were primed and boosted with pH5HA/0.3% (w/v) P85, non-targeted polyplexes and targeted polyplexes (**Figure 71**). An apparent lower level of HAI was observed in P85/HEZ008/pH5HA and P85/Mannose-

HEZ008/pH5HA vaccinated mice compared to those of non-targeted and targeted polyplexes (Figure 71).

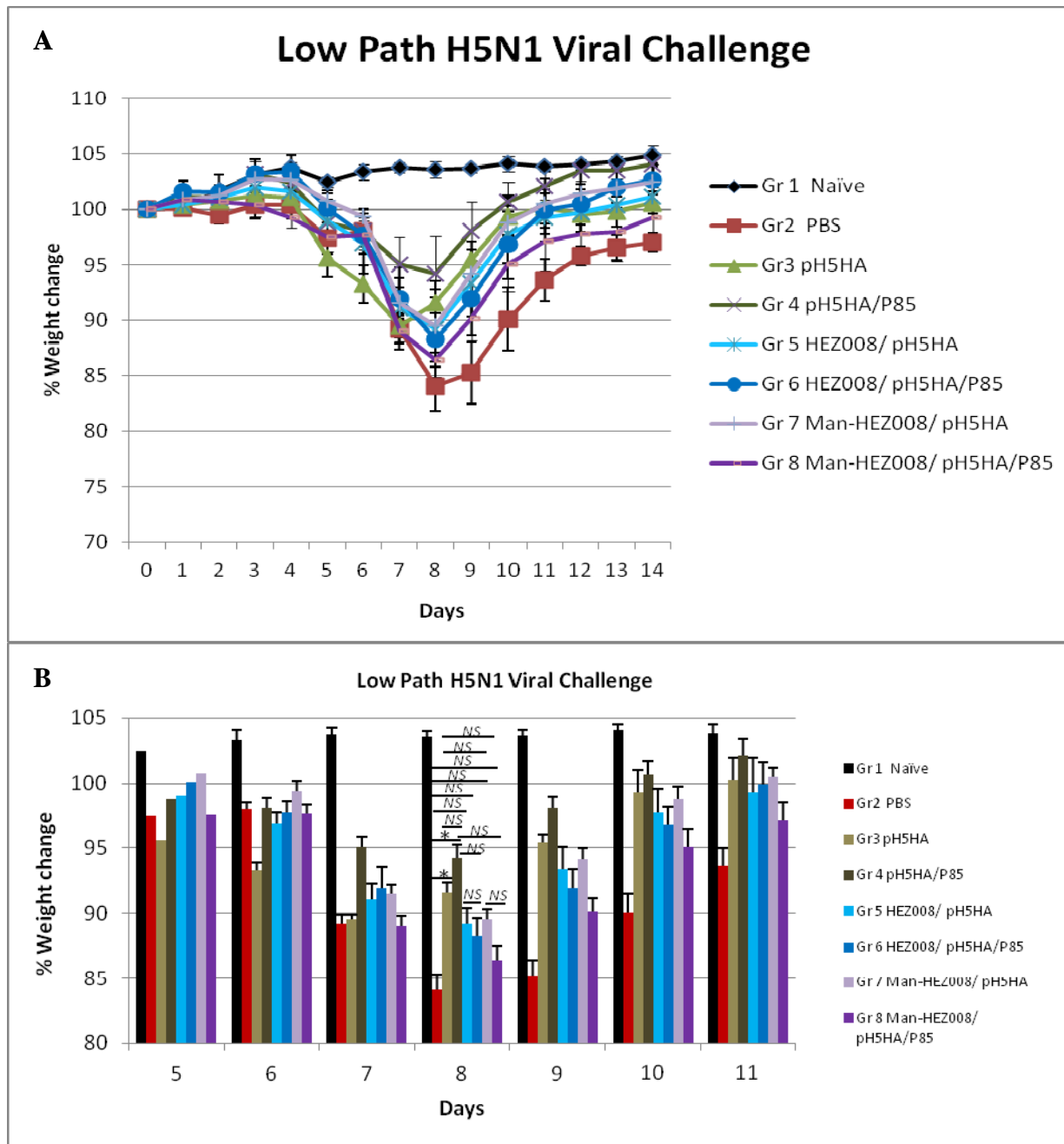


**Figure 71.** The hemagglutination inhibition (HAI) antibody response induced after pH5HA vaccination. 50  $\mu$ g of pH5HA polyplexed with non-targeted (HEZ008/pH5HA) and targeted (Mannose-HEZ008/pH5HA) at 40% mannose density and formulated with/without 0.3% w/v Pluronic P85. 50  $\mu$ L formulations in PBS were administered in tibialis anterior muscles (5 mice/group) and boosted with the same formulations on D28. Sera from individual mice were taken 56 days after administration of vaccine formulations and were used to measure HAI antibody response against A/Vietnam/1203/2004 (H5N1). The geometric mean titer obtained from 3 mice  $\pm$ SD.

Next, we determined whether immunization with non-targeted polyplexes (HEZ008/pDNA) and targeted polyplexes (Mannose-HEZ008/pDNA) vaccine formulations can protect the mice against Low Pathogenicity H5N1 viral challenges.

Animals (BALB/c mice, 5 mice/group) were vaccinated using a regiment described above (priming on day 0 and boosting on day 28). On day 64 after the first vaccination the animals were challenged via intranasal administration of 30  $\mu$ L of 1:10 diluted  $1.9 \times 10^6$  TCID<sub>50</sub> Low pathogenicity influenza virus A/H5N1 (VNH5N1-PR8CDC-RG). All 5 mice in each group were monitored for development of clinical signs and loss of body weight for 14 days (Figure 72).

All mice in the control group (PBS treatment) survived the challenges while exhibiting higher weight loss and delayed recovery compared to the vaccinated groups (Figure 72A and B). All the animals in P85/pH5HA vaccinated groups showed less discomfort and weight loss. P85/pH5HA and pDNA alone vaccinated groups of animals exhibited a weight loss of only 6% and 8% of their average pre-challenge body weight on day 8, respectively, and rapidly regained their original body weight on day 14 (Figure 72A). However, there was no significant difference in weight loss in groups vaccinated with polyplexes (either targeted or non-targeted) compared to control group. Only Pluronic/pH5HA and naked DNA vaccine formulations exhibited significant protection against the viral challenge, while there were no significant differences in weight loss among the animals vaccinated with polyplexes either in presence or absence of Pluronic P85 (Figure 72B).



**Figure 72.** 50  $\mu$ g of pH5HA polyplexed with non-targeted (HEZ008/pH5HA) and targeted (Mannose-HEZ008/pH5HA) copolymers and formulated with/without 0.3% w/v Pluronic P85. 50  $\mu$ L formulations in PBS were administered in tibialis anterior muscles (5 mice/group) and boosted with the same formulations on day 28. Mice were challenged intranasally on day 64 with 30  $\mu$ L of 1:10 diluted  $1.9 \times 10^6$  TCID<sub>50</sub> Low pathogenicity influenza virus A/H5N1 (VNH5N1-PR8CDC-RG). A. Body weight changes data expressed as a percentage of the pre-challenged body weight and presented as mean  $\pm$  SEM ( $n = 5$ ) and B, data analyzed with Student's  $t$ -test ( $n = 5$ ), \* $P < 0.05$  on day 8.

Overall, these results demonstrated that P85/pH5HA and naked DNA vaccine formulations exhibited more potent protective effect against Low pathogenicity influenza virus A/H5N1

(VNH5N1-PR8CDC-RG) compared to corresponding BIC complexes. No differences were observed between targeted and non-targeted BIC formulations.

## **KEY RESEARCH ACCOMPLISHMENTS**

### **Platform A:**

- Synthesis and purification of H5 HA antigen has been completed.
- The effect of polymer chemistry on immune cell activation has been quantified via cell surface marker expression and cytokine secretion profiles.
- Initial *in vivo* studies have established the required doses for HA administration in mice.
- Characteristics of polyanhydride nanoparticles capable to release stable full-length HA H5 protein were optimized in *in vitro* studies.
- Initial studies on BALB/c mice subcutaneous and intranasal vaccinations with the soluble forms of the H5-T with and without different traditional adjuvants were carried out. The highest amount of neutralizing activity was observed in the groups receiving a Toll like receptor (TLR) agonist as an adjuvant. Only intranasal immunizations were able to elicit antigen specific IgA antibody in the bronchio-alveolar lavage fluid.
- Soluble H5 hemagglutinin trimer (sH5<sub>3</sub>) alone or in conjunction with polyanhydride nanoparticles induced total serum anti-H5<sub>3</sub> IgG and neutralizing antibodies significantly greater than the saline control.
- All mice immunized with H5<sub>3</sub> and/or polyanhydride nanoparticle formulations were protective against a low pathogenic Influenza virus A/H5N1 (VNH5N1-PR8CDC-RG) challenge.

### **Platform B:**

- DNA plasmids encoding H5N1 HA protein, pVAX1-H5HA and plasmid encoding reporter gene for luciferase (Luc), pVAX1-Luc, have been efficiently constructed and expressed the transgenes *in vivo* post intramuscular injection as was determined by non-invasive animal optical imaging, real time RT-PCR as well as reverse-transcriptase PCR.
- Co-administration of pVAX1-Luc/pVAX1-H5HA plasmid with Pluronics block copolymers (0.01% SP1017/ 0.3% P85) significantly increased the level of transgene expression in skeletal muscle of mouse.
- Transgene expressions were also greatly prolonged and lasted for at least 50 and 28 days for Luc and H5HA gene respectively, in skeletal muscle.
- Following DNA-based vaccinations H5HA gene expressed, colocalized in APCs and translocated to the distal organs.
- Pluronic/pH5HA DNA vaccine formulations induced lasting primary antibody response.
- DNA-based vaccinations induced Th1, Th2 and Th17 cytokine responses in mice.
- Pluronic/pH5HA DNA vaccines induced serum HAI Ab against the A/Vietnam/1203/2004 (H5N1) virus.
- The mouse infectious dose of low pathogenic Influenza virus A/H5N1 (VNH5N1-PR8CDC-RG) was determined in order to perform the challenge study.

- Pluronic/pVAX1-H5HA DNA vaccine formulations significantly protected the weight loss compared to PBS control group when the animals are challenged with the mouse infectious dose of low pathogenic Influenza virus A/H5N1 (VNH5N1-PR8CDC-RG).
- Viral genetic loads in lungs of Pluronic/pVAX1-H5HA DNA vaccinated groups demonstrated protective effect of the vaccine formulations compared to PBS controlled mice on day 3 post-viral challenge. Pluronic P85/ pVAX1-H5HA DNA vaccinated groups had significantly less viral RNA in the lungs compared to PBS vaccinated mice.
- Preinjection of Pluronic P85 (0.3% and 3% wt/wt) 36 hours before DNA injection in tibialis anterior muscle further increased gene expression compared to DNA alone and DNA+P85 without pre injection *in vivo*. These results demonstrate role of infiltrating immune cells (neutrophils and macrophages) after preinjection and increasing transgene expression possibly by uptake of DNA and expression of transgenes in muscle tissue.
- Increase in transgene expression *in vivo* was further confirmed by *in vitro* studies where co-incubation of plasmid DNA with increasing concentration of P85 with macrophages for 4 hours demonstrated increase in transgene expression. This *in vitro* increase in transgene expression was optimal at 1% wt/wt P85, which was found to be up to 3 orders of magnitude compared to DNA alone.
- Presence of distal inflammation (peritonitis) significantly enhanced the muscle gene expression only when DNA was injected with Pluronic P85 and not DNA alone.
- Effect of inflammation on gene expression did not diminish though it was less pronounced when DNA and P85 were separated by space (Left and right hind limb).
- Gene expression (GFP) in ischemic muscle injected with DNA alone, DNA+P85 and DNA+SP1017 colocalized with CD11b (Macrophage/myeloid) cell marker in addition to muscle cells.
- *Ex vivo* transfected macrophages with GFP encoding DNA carried and expressed GFP protein selectively to ischemic muscles and not to non ischemic muscles of ischemic mice. Moreover, the ischemic muscles were found to express the GFP protein that implies the transfer of DNA/protein from macrophages to ischemic muscles.
- Horizontal transfer of DNA from macrophages to muscle cells was studied by in vitro model of co-culture which proved transfer of DNA from macrophages to muscle cells only in presence of pluronics.

#### **Platform C:**

- DNA plasmids were formulated into well-defined BICs using mannose-modified cationic block copolymers, mannose-PEG-PLL.
- Stability of BICs was reinforced by introduction of reductively cleavable crosslinks.
- In vitro cytotoxicity studies revealed that crosslinked BICs were less toxic compared to non-crosslinked complexes.
- Transfection efficiency was preliminarily evaluated in murine and human macrophage cells.
- Folate receptor expression was confirmed in RAW264.7 murine macrophage cells.
- Alternative formulation 1-Folate-BICs were produced and cellular uptakes as well as in vitro transfection efficiency were preliminarily evaluated.
- The synthesis of a new batch of cationic block copolymers Folate-PEG-PLL with higher purity (quality) was done.

- Alternative formulation 2-cationic liposome/DNA(lipoplexes) were produced, characterized and DDAB lipoplex showed promising transfection efficiency in human macrophage cells.
- New cationic poly(2-Oxazolines) (POx) polymers HEZ008 formed polyplexes with pDNA in spherical morphology and 100nm size by TEM imaging.
- HEZ008/DNA polyplexes, or BICs, were stable against thermal challenge and DNase I.
- The HEZ008 polymers exhibited very low cytotoxicity to RAW macrophage cells as well as B16 melanoma cells comparing to PEG-PLL.
- The HEZ008/pDNA polyplexes have very low serum protein binding and slow uptake by macrophage cells, allowing minimum non-specific cellular uptake and potential targeting delivery to selected cell population.

## CONCLUSIONS

- The robust procedures for the synthesis and purification of H5 HA antigen were developed.
- Mice receiving sH5<sub>3</sub> alone induced neutralizing antibody titers that were protective against a low pathogenic viral challenge, similar to mice receiving polyanhydride nanoparticles. These initial results display a proof-of-concept for this vaccine platform, emphasizing the strength and immunogenicity of the hemagglutinin trimer cloned and expressed.
- We demonstrated that co-administration of DNA (pVAX1-Luc and pVAX1-H5HA vaccines with Pluronic block copolymers (Pluronic P85 or SP1017) significantly enhance gene expression and prolongation of both Luciferase reporter gene and therapeutic H5HA gene in the skeletal muscle of mouse.
- In response to administration of pVAX1-H5HA DNA and pVAX1-H5HA DNA/P85 or SP1017 the APC incorporated, expressed and transported the H5HA transgene to the central immune organs. Pluronic/pVAX1-H5HA DNA vaccine formulations could induce a primary antibody response and that the level of the induced total IgG antibody was long lasting.
- Pluronic/pVAX1-H5HA DNA vaccine formulations have significant protective effect over PBS control treatment against Low pathogenicity influenza virus A/H5N1 (VNH5N1-PR8CDC-RG).
- Presence of distal inflammation and its effect on gene expression provides a novel track for pluronic gene therapy in disease models (that often involve inflammation). This would imply even higher expression of therapeutic genes in inflammation models when the therapeutic genes are driven by CMV promoters.
- We believe that Pluronic block copolymers selectively recruit antigen presenting cells (monocytes/macrophages) to muscle tissue upon DNA injection which phagocytose the plasmid DNA and then in turn transfect muscle tissue thus increasing the muscle transfection efficiency.
- The methods for the preparation of targeted, stable, and well-defined BICs based on mannose-capped poly(ethylene glycol)-block-poly(l-lysine) diblock copolymers and plasmid DNA were developed.

- Mannose functionalized BICs provide a safe and stable platform for gene delivery to macrophages.
- P85/pH5HA and naked DNA vaccine formulations exhibited more potent protective effect against Low pathogenicity influenza virus A/H5N1 (VNH5N1-PR8CDC-RG) compared to corresponding Mannose functionalized BIC complexes. No differences were observed between targeted and non-targeted BIC formulations.

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**What opportunities for training and professional development has the project provided?**

*If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”*

*Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.*

This project provided opportunities for training and professional development for graduate students and postdoctoral trainees who were involved in the activities supported by this project at the University of Nebraska Medical Center and Iowa State University. The acquired knowledge and skills will assist them for future careers in areas of basic biomedical research, translational research, industrial research and development.

**How were the results disseminated to communities of interest?**

*If there is nothing significant to report during this reporting period, state “Nothing to Report.”*

*Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.*

The results related to the polymeric nanovaccines were discussed during the lectures to the undergraduate students and professional students at the both Universities as a part of the topic related to recent advances in nanomedicine and specifically in design of new vaccines.

**What do you plan to do during the next reporting period to accomplish the goals?**  
*If this is the final report, state “Nothing to Report.”*

*Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.*

Nothing to Report.

- 4. IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

**What was the impact on the development of the principal discipline(s) of the project?**  
*If there is nothing significant to report during this reporting period, state “Nothing to Report.”*

*Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).*

The project provided a basis to exploit the advantages of nanovaccines in pandemic scenarios of avian influenza H5N1. Here, we showed that the better protection against virus can be achieved using antigen encapsulated into the polymeric carriers. It is also advanced our knowledge regarding an effects of synthetic polymers on elicited immune response.

**What was the impact on other disciplines?**  
*If there is nothing significant to report during this reporting period, state “Nothing to Report.”*

*Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.*

The vaccine development strategy used in this project will have significant impact on the field of protein/gene therapy for treating various other diseases as well. It will also make an impact on development of future nanomedicines through interdisciplinary efforts of scientists working in the areas of biochemistry, molecular biology, microbiology, pathology and immunology, drug and gene delivery, and materials science and engineering.

**What was the impact on technology transfer?**

*If there is nothing significant to report during this reporting period, state “Nothing to Report.”*

*Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:*

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

**What was the impact on society beyond science and technology?**

*If there is nothing significant to report during this reporting period, state “Nothing to Report.”*

*Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:*

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

This project increases the awareness about less known avian influenza H5N1, which will have a significant impact on socio-economic conditions. I will also facilitate the public knowledge regarding the advantages of novel vaccine development strategies compared to traditional attenuated virus vaccines.

- 5. CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:

**Changes in approach and reasons for change**

*Describe any changes in approach during the reporting period and reasons for these changes.*

*Remember that significant changes in objectives and scope require prior approval of the agency.*

Nothing to report.

**Actual or anticipated problems or delays and actions or plans to resolve them**

*Describe problems or delays encountered during the reporting period and actions or plans to resolve them.*

Nothing to report.

**Changes that had a significant impact on expenditures**

*Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.*

Nothing to report.

**Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

*Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.*

**Significant changes in use or care of human subjects**

Nothing to report.

**Significant changes in use or care of vertebrate animals.**

Nothing to report.

**Significant changes in use of biohazards and/or select agents**

Nothing to report.

**6. PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**  
Report only the major publication(s) resulting from the work under this award.

**Journal publications.** *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. Kathleen A. Ross, Lucas M. Huntimer, Julia E. Vela-Ramirez, Justin R. Adams, Susan L. Carpenter, Marian L. Kohut, Tatiana Bronich, Richard Webby, Kevin L. Legge, Surya K. Mallapragada, Michael J. Wannemuehler, Balaji Narasimhan “Vaccine technologies against avian influenza: current approaches and new directions”, *Journal of Biomedical Nanotechnology*, 2014. 10(9), 2261-2294. *Published. Acknowledgement of federal support (yes).*
2. Kathleen A. Ross, Hyelee Loyd, Wuwei Wu, Lucas Huntimer, Michael J. Wannemuehler, Susan Carpenter, Balaji Narasimhan. Structural and antigenic stability of H5N1 hemagglutinin trimer upon release from polyanhydride nanoparticles. *J Biomed Mater Res A*. 2014, Jan 17. doi: 10.1002/jbm.a.35086. [Epub ahead of print]. *Published. Acknowledgement of federal support (yes).*
3. Kathleen A. Ross, Hyelee Loyd, Wuwei Wu, Lucas Huntimer, Shaheen Ahmed, Anthony Sambol, Scott Broderick, Zachary Flickinger, Krishna Rajan, Steven Hinrichs, Tatiana Bronich, Surya Mallapragada, Michael J. Wannemuehler, Susan Carpenter, and Balaji Narasimhan. “Hemagglutinin-based polyanhydride nanovaccines against H5N1 influenza elicit protective virus neutralizing titers and cell-mediated immunity.” *Int J Nanomedicine*. Submitted (2014, in revision). *Acknowledgement of federal support (yes).*
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9. Shaheen Ahmed, Anthony Sambol, Steven Hinrichs, Alexander V. Kabanov, Tatiana K. Bronich. "The impact of Pluronic Block-copolymers on H5 hemagglutinin transgene expression, immune responses and protection against avian influenza in mice." In preparation. *Acknowledgement of federal support (yes)*.

**Books or other non-periodical, one-time publications.** *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

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**Other publications, conference papers, and presentations.** *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (\*) if presentation produced a manuscript.*

1. Kathleen A. Ross, Hyelee Loyd, Wuwei Wu, Lucas Huntimer, Shaheen Ahmed, Anthony Sambol, Scott Broderick, Zachary Flickinger, Krishna Rajan, Steven Hinrichs, Tatiana Bronich, Surya Mallapragada, Michael J. Wannemuehler, Susan Carpenter, and Balaji Narasimhan. "Hemagglutinin-based polyanhydride nanovaccines against H5N1 influenza elicit protective virus neutralizing titers and induce cell-mediated immunity." Society for Biomaterials Annual Meeting, April 16-19, 2014.\*
2. Kathleen A. Ross, Hyelee Loyd, Wuwei Wu, Lucas Huntimer, Shaheen Ahmed, Anthony Sambol, Zachary Flickinger, Steven Hinrichs, Tatiana K. Bronich, Surya K Mallapragada,



Michael J. Wannemuehler, Susan L Carpenter, and Balaji Narasimhan, "Polyanhydride nanovaccines elicit protective virus neutralizing titers and cell-mediated immunity against influenza," *Trans Soc Biomater*, 37, 538, 2014.

3. Kathleen A. Ross, Hyelee Loyd, Wuwei Wu, Lucas Huntimer, Shaheen Ahmed, Anthony Sambol, Zachary Flickinger, Steven Hinrichs, Tatiana Bronich, Surya Mallapragada, Michael J. Wannemuehler, Susan Carpenter, and Balaji Narasimhan. "Polyanhydride-based Influenza HA Nanovaccines Elicit Virus Neutralizing Titers and Enhance Cell-mediated Immunity." AICHE Annual Meeting, November 3-8, 2013.
4. Lucas Huntimer, Amanda E. Ramer-Tait, Latrisha K. Petersen, Kathleen A. Ross, Katharine A Walz, Chong Wang, Jesse M Hostetter, Michael J. Wannemuehler, and Balaji Narasimhan, "Polyanhydride nanoparticle-based influenza vaccine elicits viral neutralizing titers and enhances cell-mediated immunity", *Trans Soc Biomater*, 36, 2013.
5. Kathleen A. Ross, Shannon L Haughney, Timothy M Brenza, Lucas Huntimer, Mercedes P Boggiato, Michael J. Wannemuehler, and Balaji Narasimhan, "Deposition and persistence of polyanhydride nanoparticle vaccines upon intranasal administration", *Trans Soc Biomater*, 36, 2013.
6. Lucas Huntimer, Kathleen A. Ross, Ross J. Darling, Amanda E. Ramer-Tait, Michael J. Wannemuehler, and Balaji Narasimhan, "Humoral responses elicited by polyanhydride nanoparticle formulations are facilitated by enhanced CD4+ T cell helper cells", *Trans Soc Biomater*, 36, 2013.
7. Lucas Huntimer, Kathleen A. Ross, Ross Darling, Amanda E. Ramer-Tait, Michael J. Wannemuehler, and Balaji Narasimhan, "Polyanhydride particle vaccine platform enhances antigen-specific cytotoxic T cell responses", *Trans Soc Biomater*, 36, 2013.
8. Zhijian He, Yingchao Han, Lei Miao, Devika S Manickam, Rainer Jordan, Robert Luxenhofer, Alexander V Kabanov, Poly(2-oxazolines) as Novel Drug Delivery Systems: Synergistic Combinations of Anticancer Drug Delivery and Polyplexes for Macrophage-targeted Gene Delivery. Gordon Research Conference-Cancer Nanotechnology, Mount Snow Resort, West Dover, VT, July 14-19, 2013.
9. Shaheen Ahmed, Tatiana K. Bronich, Alexander Kabanov. "Synthetic DNA Vaccine against Avian Influenza A Virus (H5N1)." The 15th Annual Meeting of the American Society of Gene & Cell Therapy, Philadelphia, PA, May 15-19, 2012.

10. V. Mahajan, A. Kabanov, "Pluronic block copolymer as a potential gene delivery candidate", 15<sup>th</sup> Annual meeting of American Society of Cell and Gene Therapy (ASGCT), Philadelphia, PA, May 15 -19 2012.
11. Lucas Huntimer, Amanda E. Ramer-Tait, Latrisha K. Petersen, Kathleen A. Ross, Katherine A Walz, Chong Wang, Jesse M Hostetter, Michael J. Wannemuehler, and Balaji Narasimhan, "Enhanced safety profile of polyanhydride nanoparticle vaccine platforms", Trans Soc Biomater, 35, 2012.
12. Lucas Huntimer, Kathleen A. Ross, Latrisha K. Petersen, Balaji Narasimhan, Michael J. Wannemuehler, and Amanda E. Ramer-Tait, "Antigen loaded polyanhydride particle vaccines expand CD8+ T cell responses at early time points", Trans Soc Biomater, 35, 2012.
13. Jennifer H. Wilson Welder, Lucas Huntimer, Kathleen A. Ross, Brenda R. Carrillo-Conde, Lynn Pruisner, Balaji Narasimhan, and Michael J. Wannemuehler, "Encapsulation of antigens into microparticles results in dosage sparing capabilities," Trans Soc Biomater, 34, 626, 2011.
14. Kathleen A. Ross, Lucas Huntimer, Latrisha K Petersen, Michael J Wannemuehler, and Balaji Narasimhan, "Stability and Release of H5N1 Hemagglutinin Antigens Encapsulated in Polyanhydride Nanoparticles," Annual AIChE Meeting, Minneapolis, MN, October 17, 2011
15. Shaheen Ahmed, Tatiana K. Bronich, Alexander Kabanov, "Development of DNA Vaccine against Avian Influenza A Virus (H5N1)," 14th Annual Meeting of the American Society of Gene & Cell Therapy, Seattle, WA, May 18-21, 2011.
16. Shaheen Ahmed, Caroline Roques, Tatiana K. Bronich, Alexander Kabanov. "Adjuvanted DNA Vaccine against Avian Influenza A Virus (H5N1)", 8th International NanoDDS, Omaha, NE, Oct. 2010.

#### Invited Presentations

Presented by Balaji Narasimhan

1. "Pathogen Mimicking Nanovaccine Platform Technology: A New Paradigm," Department of Chemical and Biological Engineering, Colorado State University, Fort Collins, CO, September 5, 2014
2. "Pathogen Mimicking Nanoparticles for Prevention and Treatment of Respiratory Infectious Diseases," Department of Chemical Engineering, Texas Tech University, Lubbock, TX, April 5, 2013

3. "Pathogen Mimicking Nanovaccine Platform Technology: A New Paradigm," 247th ACS National Meeting, Dallas, TX, March 19, 2014
4. "Pathogen Mimicking Nanoparticles for Prevention and Treatment of Respiratory Infectious Diseases," New Cells, New Vaccines VII: From Protein to Product, Wilmington, DE, March 19, 2013
5. "Pathogen Mimicking Nanoparticles for Prevention and Treatment of Respiratory Infectious Diseases," School of Chemical Engineering, Purdue University, West Lafayette, IN, October 18, 2012
6. "Pathogen Mimicking Nanoparticles for Prevention and Treatment of Respiratory Infectious Diseases," Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE, October 8, 2012
7. "Nanoparticle-based Platform Vaccines for Respiratory Infectious Diseases," DTRA Chemical and Biological Defense Science and Technology Conference, Las Vegas, NV, November 17, 2011
8. "Pathogen Mimicking Nanoparticles for Prevention and Treatment of Respiratory Infectious Diseases," Mystic Pharmaceuticals, Inc., Austin, TX, November 2, 2011
9. "Pathogen Mimicking Nanoparticles for Prevention and Treatment of Respiratory Infectious Diseases," Area 15 Plenary Lecture, Annual AIChE Meeting, Minneapolis, MN, October 18, 2011
10. "Single Dose Nanoadjuvant Platform Vaccines Against Biowarfare Agents," Center for Drug Delivery and Nanomedicine Retreat, University of Nebraska Medical Center, Nebraska City, NE, June 1, 2011
11. "Pathogen-Mimicking" Nanoparticles for Prevention and Treatment of Respiratory Infectious Diseases," California Nanosystems Institute, UCLA, Los Angeles, CA, May 3, 2011.
12. "Pathogen Mimicry: A Viable Design Strategy for Prevention and Treatment of Respiratory Infectious Diseases," Osborn Club, Iowa State University, Ames, IA, April 11, 2011.
13. "Nanoparticle-Based Platforms for Biodefense Pathogens and Global Public Health," Global Biosurveillance: Enabling Science and Technology, 2nd Biothreat Nonproliferation Conference, Santa Fe, NM, January 19, 2011.

14. "Nanovaccine Platforms for Infectious Respiratory Diseases," FAN-IITB Conference on Biosciences and Bioengineering Applications in Healthcare, Santa Clara, CA, September 26, 2010.
15. "Nanovaccine Platforms for Biodefense Pathogens," Department of Chemical and Biochemical Engineering, University of Iowa, Iowa City, IA, September 16, 2010.

- **Website(s) or other Internet site(s)**

*List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.*

Nothing to report.

- **Technologies or techniques**

*Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.*

Nothing to report.

- **Inventions, patent applications, and/or licenses**

*Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.*

No inventions or patent applications resulted from the project.

- **Other Products**

*Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:*

- *data or databases;*
- *biospecimen collections;*
- *audio or video products;*

- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

No other product were developed.

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

### What individuals have worked on the project?

*Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change.”*

#### Example:

*Name:* Mary Smith  
*Project Role:* Graduate Student  
*Researcher Identifier (e.g. ORCID ID):* 1234567  
*Nearest person month worked:* 5

*Contribution to Project:* Ms. Smith has performed work in the area of combined error-control and constrained coding.  
*Funding Support:* The Ford Foundation (Complete only if the funding support is provided from other than this award).

Tatiana Bronich (no change)  
 Shaheen Ahmed (no change)  
 Jamie Arbaugh (no change)

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

*If there is nothing significant to report during this reporting period, state “Nothing to Report.”*

*If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.*

**Renewed: 2 P20 GM103480-06 (Bronich) NIH / NIGMS “COBRE: Nebraska Center for Nanomedicine”**  
09/15/2013 – 05/31/2018

**Awarded: HDTRA1-13-C-0037 (V. Gilman (Vivonics), T. Bronich (UNMC)) U.S. Army subcontract from Vivonics, Inc. “Highly Stable Human Plasma Butyrylcholinesterase for Intra-Muscular Delivery”** 09/09/2013 – 09/08/2014

**Ended: BC102673P1 (Collaborative , PIs: Band and Bronich) U.S. Army” Mechanism-based Enhanced Delivery of Drug-loaded Targeted Nanoparticles for Breast Cancer Therapy”**  
01/18/2011 - 01/17/2014

**What other organizations were involved as partners?**

*If there is nothing significant to report during this reporting period, state “Nothing to Report.”*

*Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.*

*Provide the following information for each partnership:*

Organization Name:

Location of Organization: (if foreign location list country)

Partner’s contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner’s facilities for project activities);*
- *Collaboration (e.g., partner’s staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner’s staff use each other’s facilities, work at each other’s site); and*
- *Other.*

Nothing to report.

## **8. SPECIAL REPORTING REQUIREMENTS**

**COLLABORATIVE AWARDS:** For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.

**QUAD CHARTS:** If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.

- 9. APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.